STUDIES ON STRUCTURE OF WHEAT GLUTENIN(Dissertation_全文)

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CONTENTS

Abbreviations

CM-Cys	S-carboxymethyl cysteine
CM-Glutenin	reduced and carboxymethylated glutenin
EDTA	ethylenediaminetetraacetic acid
HMW-Subunits	subunit polypeptides in high molecular weight region
IAM	Iodoacetamide
LMW-Subunits	subunit polypeptides in low molecular weight region
2-ME	2-mercaptoethanol
MW	molecular weight
PE-Cys	S-pyridylethyl cysteine
PE-Glutenin	reduced and pyridylethylated glutenin
SDS	sodium dodecylsulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SH	sulfhydryl
SS	disulfide

CHAPTER I

General Introduction

Wheat has been prized by man since ancient times and is today the world's largest cereal.[1,2] Demand for wheat is very large, because among all the cereal grains only wheat yields such flour as to produce "elastic cohesive dough" when moistened and mixed.[3] Owing to such "dough forming" property, wheat flour is able to made into bread, noodle products, etc. It has been very important subject for cereal scientists to elucidate which components of wheat flour is responsible for the unique functional property of wheat flour dough. Nowadays, the unique properties of wheat flour dough seem to be due to the water-insoluble protein, that is, "gluten". This conclusion is evidenced by the following facts; First,after one extracts lipids and washes away starch, water-soluble proteins (albumin + globulin) and other components from wheat flour, one can still retain a hydrated "rubbery mass", gluten, which is 80 % protein.[4,5] Second , the bread-making quality of wheat flour is closely relating to the gluten content of each flour. In general, the "harder wheats" that are better for making bread give flours

that contain more gluten.[1,6-11] Third, one can reconstitute flour to produce a cohesive dough from gluten protein with starch, lipids and other components derived from other cereals.[12-14]

Gluten is composed of almost equal amount of gliadine (the protein fraction which is soluble in 70 % ethanol) and glutenin (the fraction which is insoluble in 70 % ethanol, but soluble in acidic or alkaline solvents)[16]. It is thought that gliadine is the mixture of many kinds of polypeptide monomers,[17-19] whereas glutenin is a large molecular weight protein consisting of subunit polypeptides polymerized or associated.[19-21] Concerning the roles of these two proteins in dough-forming, gliadine seems to provide appropriate fluidity, consequently extensibility to gluten matrix,[22,23] and interact with other components (lipids, carbohydrates etc.) in wheat flour dough.[24] On the other hand, glutenin probably forms the fundamental skeleton of gluten matrix and primarily contributes to the occurrence of unique viscoelasticity of wheat flour dough.[22,23,25]

In spite of such important role of glutenin in dough-forming, the structure of glutenin, in other words, the mode of linkage of subunit polypeptides is

not yet known. First problem is what kinds of bonds contribute to the linkage of subunit polypeptides of glutenin. Even if glutenin is treated with the denaturants or surface-active reagents such as urea, guanidine hydrochloride or SDS, subunit monomers are not liberated [26,27] and molecular weight of glutenin is still ranging from ten thousands to millions.[20,28-30] However, if glutenin is treated with reducing agents such as 2-mercaptoethanol or cysteine, the molecular weight of glutenin dropped markedly[20] and the liberation of subunit monomers are recognized.[21] Further, the reduction of glutenin causes the increase of it's solubility,[31] and the decrease of it's viscosity in solution.[20] These observations strongly suggest that glutenin is a polymer consisting of subunit polypeptides linked through SS bonds. Nevertheless, there were still several reports,[1, 32-34] claiming that glutenin sububnits are not polymerized through SS bonds, but only aggregate through non-covalent forces. Hence, at present, it is not concluded yet completely which kind of bond really contributes to the linkage of subunit polypeptides, SS bond or non-covalent bond , although there were no reliable evidence suggesting the contribution of non-covalent forces.[28] Second problem

is how each subunit is linked to other subunits through SS bonds, assuming that glutenin is a polymer consisting of subunit polypeptides linked through SS bonds. Ewart[35] showed that the evidence that glutenin subunits have both inter- and intra-polypeptide SS bonds and proposed that subunit polypeptides linked "linearly" through two inter-polypeptide SS bonds. Bietz[36] and Wall[22] advanced the net work structure in which each subunit polypeptide is linked to more than three subunits through inter-polypeptide SS bonds. Although there has been so far many studies,[37-49] it has not been determined which structural model of glutenin is valid or not.

As mentioned above, the structure of glutenin is almost obscure. However, in order to understand the mechanism of occurrence of unique viscoelasticity during dough-forming, it is necessary to establish the whole structural model of gluenin. Thus, in this thesis, in order to elucidate the whole structure of glutenin molecule, the author carried out the following investigations : First, the author examined the method to separate subunit polypeptides and determined total cysteine contents of separated subunits. Second, in order to determine how many numbers of total cysteine

residues in each subunit is responsible for the inter-polypeptide SS bonds, the condition for the selective cleavage of inter-polypeptide SS bonds were investigated. Third, inter- and intra-polypeptide SS bonds of each subunit were determined separately by using the chemical modification methods. The amount of inter-polypeptide SS bonds of each subunit thus determined revealed that glutenin is a polymer consisting of subunit polypeptides linked "linearly" through two inter-polypeptide SS bonds. Fourth, in order to investigate subunit-subunit interaction through noncovalent forces, gel filtration of reduced and alkylated glutenin was carried out in the presence or absence of urea. Finally, from the results obtained in this thesis, the author proposed the whole structural model of glutenin molecule including subunit-subunit interaction through both SS bonds and non-covalent forces.

CHAPTER II

Separation of Subunit Polypeptides of Glutenin by SDS-PAGE and Determination of Their Cysteine Contents

I-1 INTRODUCTION

Glutenin seems to be a polymer protein consisting of many kinds of subunit polypeptides which are linked to one another through SS bonds[10]. Glutenin may have intra-polypeptide SS bonds in addition to inter-polypeptide ones. It is necessary to determine the number of inter- and intra-polypeptide SS bonds in subunits separately in order to understand how glutenin subunits are linked. Before determination of inter- and intra-polypeptide SS bonds, we must separate the main glutenin subunits and determine the total SS bonds in them. A few reports have just been concerned with separation of glutenin subunits. Huebner and Wall.[50, 51] tried to separate subunit polypeptides by gel-filtration and ion-exchange chromatography, and determine their Cys contents. However, although some subunit fractions they obtained were very homogeneous on starch-gel electrophoresis and SDS-PAGE, it was found to be difficult to separate all main subunits simply by using these methods. On the other hand, SDS-PAGE

was a good method to separate the main subunits according to their molecular weights. Danno et al.[52] and Arakawa et al.[53] used SDS-PAGE for separation of glutenin subunits. But both groups didn't determine the Cys contents of the separated subunits. Hence in this chapter,[54] we tried to separate the main subunit polypeptides of glutenin by SDS-PAGE and determine the amount of Cys in each subunit by amino acid analysis.

Danno et al.[52] reported the amino acid compositions of glutenin subunits which were separated by SDS-PAGE. However, several differences in the amounts of amino acids were observed between the results of Danno et al. and those in the present chapter. It is thought that these differences are due to the methods of hydrolysis. That is, although we extracted proteins from gel and hydrolyzed them, they hydrolyzed subunit polypeptides in the gel without extraction. Hence, we investigated the influence of gel on the amino acids when proteins were hydrolyzed in the gel without extraction.

II-2 MATERIALS AND METHODS

Materials.

Wheat flour (60 % extracts of NO.1 CW wheat milled with a test mill) was provided by Nisshin Flour

Milling Co., Ltd., Kobe. Reagents were obtained from Wako Chemical Ind. Ltd. or Nakarai Chemical Ltd.

Preparation of Glutenin.

Wheat flour was defatted with 10 vol of n-butanol at room tenperature for 12 hr and the solvent was removed by air-drying. The defatted flour was made into a dough and hand-kneaded thoroughly in 1 % NaCl solution at 0°C to remove starch and water soluble proteins. The wet gluten thus obtained was dispersed in 0.01 M acetic acid and centrifuged at 20,000 x g for 30 min. The supernatant was heated in boiling water for 15 min to inactivate proteases.[55)]

Crude glutenin was separated from the gluten solution by the alcohol fractionation described by Woychik et al.[56)] Glutenin was further purified with CM--Sephadex according to the method described by Mita et al.[57)] Glutenin thus obtained was lyophilized and stored for use.

Reduction and Pyridylethylation of Glutenin.

Reduction of SS bonds of glutenin and pyridylethylation of SH groups were carried out according to the methods of Friedman et al.[58)]

Analytical SDS-PAGE.

SDS-PAGE was carried out on a slab gel (1 x 160 x 120 , mm) using the discontinuous buffer system of Laemmli.[59] Electrophoresis was performed at 20 mA/slab for 4-5 hr at 20°C. After being stained with 0.1 % Coomassie blue R-250 in methanol-water-acetic acid (5 : 4 : 1, v/v) for 20 min, the gel was destained in methanol-water-acetic acid solution (10 : 83 : 7, v/v) with continuous shaking, and dried on filter paper.

Preparative SDS-PAGE.

Preparative SDS-PAGE was carried out on a thick slab gel (5 x 210 x 150, mm) using the same buffer system as used for analytical SDS-PAGE. Electrophoresis was performed at 20 mA/slab for 16-20 hr at 20°C.

Extraction of Subunit Polypeptides from Gel after Preparative SDS-PAGE.

After electrophoresis, the preparative SDS-PAGE gel was soaked in methanol-water-acetic acid solution (10 : 83 :7, v/v) with continuous shaking until the protein became visible as turbid white bands on removal SDS from the gel. The visible bands were cut out with a razor blade, and each gel fragment was

crushed into small pieces. A part of the crushed gel was hydrolyzed with 6 N HCl and submitted to amino acid analysis (described later). The rest of the crushed gel was soaked in 0.125 M Tris-HCl buffer, pH 6.8, containing 4 % SDS and 20 % glycerol. The suspension was stirred for 12 hr and heated at 100°C for 5 min to ensure binding of SDS to the protein.

The electrophoretic extraction of protein from gel was carried out with a glass tube (20 mm diameter) according to the electrophoretic elution method.[60] Extraction was performed at 40 mA for 16-20 hr at 20°C using the buffer system of Laemmli.[59]

Amino Acid Analysis.

Proteins and proteins in gel were hydrolyzed with 6 N HCl in sealed and evacuated tubes at 110°C for 22 hr. Amino acid analysis was performed with an analyzer (Hitachi KLA-5). Amounts of Cys were determined as PE-Cys.

II - 3 RESULTS AND DISCUSSION

Separation of Glutenin Subunits by SDS-PAGE.

Glutenin was reduced and alkylated with vinylpyridine. PE-glutenin thus obtained was subjected to

preparative SDS-PAGE. After electrophoresis each band observed in the gel was cut out separately. Subunit polypeptides extracted from the gel pieces, were analyzed by SDS-PAGE in order to examine their homogeneity.

The results are shown in Fig. II-1. The electrophoretic pattern of the whole glutenin is shown in the right column with designation of bands of main subunit polypeptides from 1 to 7. The molecular weights shown were obtained from electrophoretic mobilities. Since B-1 and B-1' appeared very closely in the electrophoretic pattern, they were separated together. The separation was successful for the purpose of the study, al-

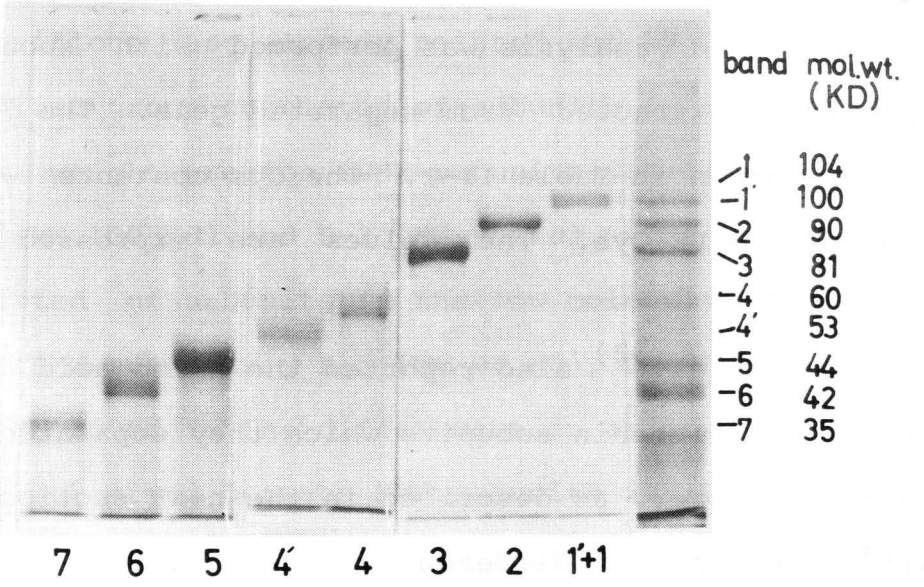


Fig. II-1. SDS-PAGE of the Separated Subunit Polypeptides of Glutenin.
B-1 (1 + 1') through 7 subunit polypeptides, separated from preparative SDS-PAGE gel, were solubilized in SDS buffer of the Laemmli system without 2-ME and submitted to SDS-PAGE. The pattern of complete reduction of glutenin is shown in the right column. The gel concentration was 7.5 %.

though a trace of contamination was observed in B-4, 5, 6 and 7. Separation of subunit polypeptides of glutenin by SDS-PAGE was tried by Danno et al.[52] and Arakawa et al.[53] Danno et al. employed the buffer system of Weber and Osborne ,[60] and Arakawa et al. used the method described by Hamauzu et al.[61] We employed the buffer system of Laemmli[59] in the present study, which resulted in better resolution of electrophoretic bands.

Amino Acid Compositions of Subunit Polypeptides of Glutenin.

Amino acid analysis was performed with subunit polypeptides extracted from separated gels. The results are shown in Table II-I. The Cys contents were determined as PE-Cys. The values are expressed as mole %.

Danno et al.[52] also reported the amino acid compositions of glutenin subunits which they separated by SDSPAGE. However, as described in the next section in detail, significant differences were observed between our results (Table II-I) and theirs. Nevertheless, in the present study, glutenin subunits were divided into three groups based on the similarity of amino acid

Table II-I Amino Acid Composition of Subunit Polypeptides of Glutenin Extracted from Gels*

Amino acid	B-1	B-2	B-3	B-4	B-4'	B-5	B-6	B-7
Lys	5.5	3.7	3.7	5.9	5.2	3.4	2.9	2.4
His	1.9	1.6	1.9	2.4	2.4	2.4	1.9	1.6
Arg	1.8	1.7	2.3	2.9	1.7	2.3	2.4	2.3
Asp	3.7	4.6	3.9	5.9	6.3	3.2	3.6	3.6
Thr	3.5	3.6	3.8	3.4	3.3	3.2	3.2	3.2
Ser	12.5	10.8	11.1	12.4	13.0	9.7	9.6	9.0
Glu	23.6	23.9	24.4	21.8	24.0	28.6	27.2	27.7
Pro	8.5	8.5	9.6	5.8	7.0	13.0	13.7	14.1
Gly	19.1	18.8	18.6	14.9	15.6	8.0	8.2	8.4
Ala	4.7	4.0	4.3	5.0	5.4	3.7	4.0	3.9
Val	2.9	3.3	3.4	4.0	3.6	5.1	5.1	4.9
Met	0.6	0.8	0.6	1.1	0.6	1.0	1.2	1.1
Ile	1.7	2.2	2.0	3.3	2.8	3.3	3.9	4.0
Leu	4.2	4.8	4.7	5.1	4.1	7.1	7.2	7.1
Tyr	3.6	4.6	4.0	2.4	1.9	1.5	1.9	1.4
Phe	1.3	1.7	1.4	3.5	3.1	4.0	3.8	3.5
PE-Cys**	0.5 (4.6)	0.5 (4.0)	0.7 (5.0)	tr	tr	1.1 (4.3)	1.1 (4.1)	1.4 (4.3)

* Values shown are expressed as number of residues per 100 total residues. Trp was not determined.
** Cys content was determined as PE-Cys. Values in parenthesis represent number of residues of PE-Cys per mol protein.

compositions as already pointed out by Danno et al. The first group consisted of subunits in the high molecular weight region (HMW-subunits), B-1 (MW 104 Kd), B-2 (MW 100 Kd) and B-3 (MW 90 Kd). The second group consisted of subunits in the low molecular weight region (LMW-subunits), B-5 (MW 44 Kd), B-6 (MW 44 Kd) and B-7 (MW 35 Kd). The differences in amino acid compositions observed between HMW subunits and LMW ones were already shown by Danno et al. But several other differences are newly indicated in Table II-I. First, the Cys content was 0.5-0.7 % in HMW subunits and 1.1-1.4 mol % in LMW ones. Pro contents were very higher in LMW

subunits than in HMW subunits, although Danno et al. showed that there were little differences in Pro contents between the two groups. Ser contents were slightly higher in HMW subunits than in LMW ones. These differences that are pointed out in Table II-I support classification of subunits into HMW and LMW subunits groups.

The third group consisted of B-4 (MW 60 Kd) and B-4' (MW 53 Kd). Asp contents were higher and Pro contents were lower in this group than in other glutenin subunits. Only traces of PE-Cys were observed in these subunits. As glutenin subunits seem to be linked through SS bonds, B-4 and B-4' may not be glutenin subunits.

The Influence of Gel on Amino Acid Analysis When Proteins Are Hydrolyzed in Gel.

As described above, there were significant differences between our results (Table II-I) and those of Danno et al.[52] It is thought that such differences were probably due to the different methods of hydrolysis. That is to say, although we extracted proteins from the gel and hydrolyzed them, they hydrolyzed the subunit polypeptides in the gel without extraction. But it is possible that the kind of fluor used in each

expereiment also caused such differences. Thus, in the present chapter, while analyzing the subunit polypeptides extracted from gel, we also hydrolyzed polypeptides in the gel and analyzed them. The results are shown in Table II-II. When we hydrolyzed the subunit polypeptides in gel, excess ammonia from the hydrolyzate interfered with the determination of bacic amino acid. Hence, in order to remove excess ammonia, we adjusted the pH of the hydrolyzate to 8.0 with NaOH followed by evacuation overnight. In spite of the success in removing ammonia, Pro was not detected. Other amino acids were not affected. Because of this

Table II-II Amino Acid Composition of Subunit Polypeptides of Glutenin in the Gels*

Amino acid	B-1	B-2	B-3	B-5	B-6	B-7
Lys	0.3	0.3	0.3	0.4	0.3	0.3
His	1.2	1.3	1.2	1.2	1.2	1.2
Arg	3.7	4.0	3.9	2.4	3.7	3.7
Asp	1.7	1.6	1.7	2.2	2.9	3.0
Thr	2.9	3.9	3.6	3.3	4.3	3.3
Ser	6.1	9.8	7.7	9.7	9.6	10.1
Glu	35.2	35.1	35.4	34.9	33.8	33.7
Pro**	14.0	14.0	14.0	14.0	14.0	14.0
Gly	17.0	13.5	14.4	3.6	4.1	2.4
Ala	3.3	3.5	3.6	3.0	4.0	3.8
Val	2.4	2.0	2.8	4.8	4.9	4.7
Met	0.4	0.6	0.7	1.5	1.6	2.0
Ile	1.1	1.0	1.2	3.0	3.3	3.5
Leu	5.0	3.6	4.4	8.4	8.0	7.9
Tyr	4.2	4.6	3.8	1.0	1.1	0.8
Phe	0.8	1.0	0.9	4.9	4.5	4.7

* Values shown are expressed as number of residues per 100 total residues. Trp and Cys were not determined.

** Pro was not detected in our experiment and the same value 14.0 was uniformly assumed in all subunits.

loss of Pro, the same value of 14.0 was uniformly assumed for the subunits.

The amino acid compositions of glutenin subunits in Table II-II are very similar to those of Danno et al.[52] except Lys and Arg. This suggested that the differences observed between the results in Table II-I and those of Danno et al.[52] were not due to the different kinds of wheat flour used in tne experiments but due to the different methods of hydrolysis. Hence, it may be elucidated which amino acid content changes in the presence or absence of gel, by comparing Table II-I with Table II-II. Lys, His, Asp, Ser and Gly contents are lower in the polypeptides hydrolyzed in gel (Table II-II) than the polypeptides extracted from the gel (Table II-I). It is thought that these amino acids were damaged by the hydrolysis in the presence of the gel. Thus Glu contents seem to be relatively higher in Table II-II than in Table II-I. In order to exsamine whether such amino acids are really affected by gel when proteins are hydrolyzed in the gel, we heated an amino acid mixture (amino acid standard solution, H type) and PE-glutenin in the presence of gel with 6 N HCl in a sealed and evacuated tube at 110°C for 22 hr. These expereiments suggested that although Gly was

scarcely affected, 20-50 % of Lys, His, Asp and Ser were lost on hydrolysis with gel. Furthermore, we compared our results with those of Arakawa et al.[53] who extracted some glutenin subunit polypeptides from SDS-PAGE gel and analyzed their amino acid compositions. The results of Arakawa et al. are similar to those in Table II-I and different from those in Table II-II and those of Danno et al.[52] Thus it is thought that Lys, His, Asp and Ser are easily damaged by HCl hydrolysis in polyacrylamide gel and consequently the amino acid values in Table II-I are correct.

Residual Numbers of Cys Existing in Glutenin Subunits.

By using the mol % values and MW of each subunit, we calculated the residual numbers of Cys per mole protein for each subunit. The MW used for the calculation was determined from the mobility on SDS-PAGE, shown in Fig. II-1. The residual number of Cys per mol protein is shown in parenthesis in Table II-I. Nearly the same amount of Cys, about 4 mole Cys per mole protein, was obtained for each subunit.

Huebner and Wall[50,51] separated several subunit polypeptides by gel-filtration and ion-exchange chromatography and determined their Cys contents. The

amount of Cys which they found were 5-8 mol per mol protein, that is, greater than the amounts shown in the present study. Of course, it is possible that subunit fractions separated by SDS-PAGE in the present chapter contained polypeptides which have no Cys. However, it is also thought that these differences were due to the different kinds of wheat flour. The total Cys content of whole glutenin prepared in the present chapter was 1.5 mol % , but on the contrary, it was 1.8 mol % in the results of Huebner and Wall.

In the present chapter, 4 Cys residues per molecule of each subunit was obtained. If glutenin subunits are assumed to have no SH groups, each subunit contains 2 moles of SS bonds per molecule protein. Thus, in future, it will be necessary to determine the number of inter- and intra-polypeptide SS bonds in subunits in order to determine if glutenin subunits are linked linearly or branching in some directions here and there.

CHAPTER III

Liberation of Subunit Polypeptides of Glutenin by Partial Reduction at pH 6.0

III-1 Introduction

Glutenin seems to be a polymer consisting of many kinds of subunit polypeptides which are linked to one another through SS bonds.[20] In the previous chapter,[54] I separated the main subunits of glutenin by preparative SDS-PAGE and showed that each main subunit contained about 4 Cys residues. These Cys residues seem to consist of inter- and intra-polypeptide SS bonds. Studies on glutenin subunits have been performed so far by using subunits obtained by the complete reduction of glutenin SS bonds.[50-53,57,63,64,] However, it is desirable to use subunits retaining intra-polypeptide SS bonds in order to elucidate the structure and behaviour of glutenin molecule. Yoshida et al.[65] observed that some of the SS bonds of glutenin could be reduced more rapidly than others, and they suggested the possibility of the selective cleavage of the inter-polypeptide SS bonds of the glutenin subunit. In this chapter,[66] in order to examine this possibility more exactly, I reduced glutenin with var-

ious concentrations of 2-ME at pH 6.0, and observed the liberation of subunits by SDS-PAGE. The amounts of SH groups and SS bonds in the separated subunits were also determined.

III-2 Materials and Methods

Materials.

Wheat flour (60 % extract of No. 1 CW wheat milled by a test mill) was provided by the Nisshin Flour Milling Co., Ltd., Kobe. Iodoacetamide was purchased from the protein Foundation. Other reagents were obtained from Wako Chemical Ind.,Ltd. or Nakarai Chemicals Ltd.

Preparation of Glutenin.

Glutenin was prepared from wheat flour according to the method as described in chapter II-2.

Reduction and Alkylation.

Partial reduction of the SS bonds of glutenin was carried out as follows: Glutenin was dispersed in 0.01 M acetic acid in a protein concentration of 0.04 % and de-aired by N_2 gas. 2-ME was added to the glutenin solution in various concentrations and reduction was started by raising the pH to 6.0. With the raising of the pH, glutenin quickly aggregated. After reduction

at 20°C for 15 min, twice a molar excess of vinylpyridine, compared with SH groups originated from glutenin and 2-ME, was added and alkylation (pyridylethylation) was carried out at 20°C and pH 6.0 for 16 hr. In order to avoid incomplete modification of SH groups, about 20 % more vinylpyridine was added to the reaction mixture followed by the addition of urea to 6 M. After being stirred for 1 hr, the solution was dialyzed against 0.1 M acetic acid and lyophilized.

Complete reduction of SS bonds of glutenin and carboxymethylation of SH groups was carried out as follows: 1 % glutenin solution in 0.1 M phosphate buffer, pH 8.0 containing 1 mM EDTA and 8 M urea was de-aired by N_2 gas bubbling for 20 min. After reduction by 5 mM 2-ME at 20°C for 4.5 hr, SH groups were alkylated by 6.5 mM iodoacetamide at 20°C for 16 hr in the dark. The solution was dialyzed against 0.1 M acetic acid and lyophilized.

SH Determination by Amino Acid Analysis.

S-Alkylated glutenin and glutenin subunits were hydrolyzed with 6 N HCl in sealed and evacuated tube at 110°C for 22 hr. Cys residues in the hydrolyzate were determined as CM-Cys and PE-CYs by an amino acid ana-

lyzer (Hitachi KLA-5).

Analytical SDS-PAGE.

Analytical SDS-PAGE, staining and destaining of gel was carried out as described in chapter II-2. The gel dried on a filter paper was subjected to a densitometric determination with a Shimadzu Dual-Wavelength Scanner CS-90 at a reference wavelength of 750 nm and a sample wavelength of 590 nm.

Preparative SDS-PAGE.

Preparative SDS-PAGE was carried out as described in chapter II-2.

Extraction of Subunit Piolypeptides from Gel of Preparative SDS-PAGE.

After electrophoresis, the preparative SDS-PAGE gel was soaked in a methanol-water-acetic acid solution (10 : 83 : 7, v/v) with continuous shaking until the protein became visible as turbid white bands with the removal of SDS from the gel. The visible bands were cut out with a razor blade and crushed to small pieces. The crushed gel was stirred in 0.125 M Tris-HCl Buffer,

pH 6.8 , containing 4 % SDS and 20 % glycerol for 12 hr and heated at 100°C. The electric extraction of protein from the gel was carried out with a glass tube (20 mm diameter) according to the method of electrophoretic elution.[60] The extraction was performed at 40 mA for 16-20 hr at 20°C using the buffer system of Laemmli.[59]

III-3 RESULTS AND DISCUSSION

Liberation of SH Groups from Glutenin by Partial Reduction with Various Concentrations of 2-ME.

Glutenin was reduced in the presence of various concentrations of 2-ME for 15 min and the SH groups liberated by the reduction were alkylated with vinylpyridine . Partially reduced pyridylethyl-glutenin (PE-glutenin) thus obtained was submitted to amino acid analysis to determine SH groups liberated by the treatment with 2-ME as PE-Cys. Fig. III-1 shows the relationship between the liberated SH groups of protein and the concentration of 2-ME, where the amount of SH groups is given as a percentage of the total SH groups in the completely reduced glutenin. The glutenin used in the present chapter contained 10.6 mol SH per 10^5g protein in the completely reduced state.

The results of Fig. III-1 show that, while the

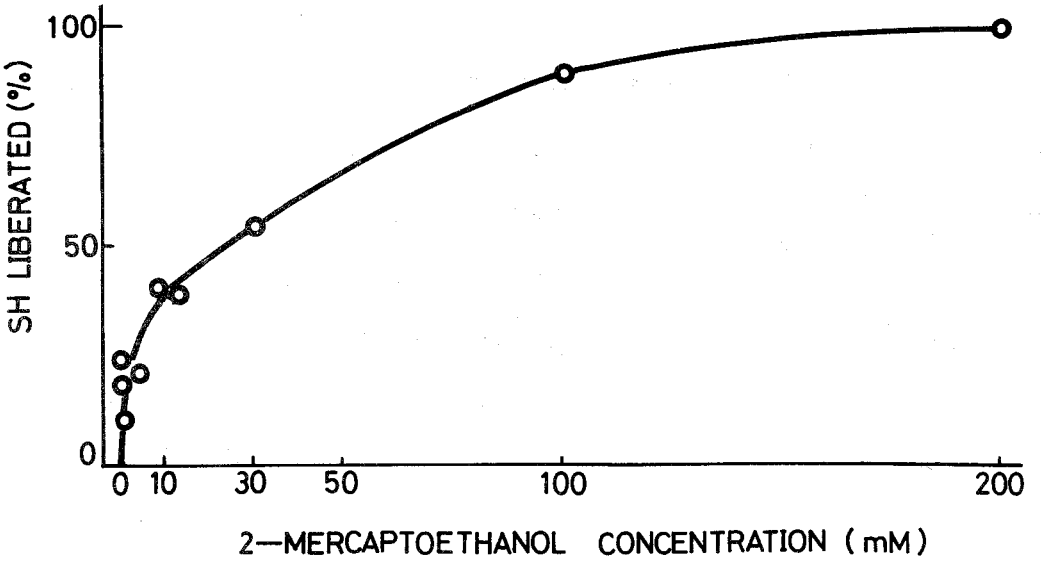


Fig. III-1. Liberation of SH Groups by Partial Reduction of Glutenin with Various Concentration of 2-ME.

SH groups liberated by the partial reduction was alkylated with vinylpyridine and determined as PE-Cys by amino acid analysis. Amounts of SH groups are given as a percentage of the total SH groups after the complete reduction of glutenin SS bonds.

amount of SH groups increased steeply with the increase of 2-ME concentration around 1 mM 2-ME, the increase declined at higher concentration to give a plateau at 6 mM. The appearance of this kind of plateau was already pointed out by Yoshida et al.[65] With further increase of 2-ME concentration, the increase of SH groups increased again to some extent and nearly all the SS bonds of protein were reduced at a 2-ME concentration of 200 mM. The observations described above show that although all the SS bonds of glutenin can be involved in the SH-SS interchange reaction, their reactivities are not uniform. That is to say, some of the

SS bonds are highly reactive ones that are reduced easily with low concentrations of 2-ME, but others are less reactive ones whose reactivity with 2-ME is restricted. The appearance of a plateau means the consumption of highly reactive SS bonds, hence the level of the plateau may correspond to the amount of highly reactive SS bonds. The level of the plateau was 40 % in this chapter, while it was 25 % in the results of Yoshida et al.[65] This difference could be explained by the difference of the protein concentration of glutenin solution used. The protein concentration was 0.04 % in this chapter , while it was 1 % in the study of them.[65] High protein concentration may accelerate the aggregation of protein, resulting in reducing the reactivity of SS bonds with 2-ME by confining them in aggregates.

Liberation of Subunit by Partial Reduction of Glutenin.

Subunit monomers of glutenin should be liberated when inter-polypeptide SS bonds are cleaved by the partial reduction. Therefore, the liberation of subunits was investigated by SDS-PAGE with PE-glutenin obtained by partial reduction with various concentrations of 2-ME. The electrophoretic patterns obtained

are shown in Fig. III-2. It includes the pattern of the complete reduced PE-glutenin, which gives major bands at the positions of MW 104 Kd, 100 Kd, 90 Kd, 81 Kd, 60 Kd, 53 Kd, 44 Kd, 42 Kd, and 35 Kd. They are designated as Bands 1, 1', 2, 3, 4, 4', 5, 6, and 7, respectively. With partial reduction, Bands 1, 1', 2, 3, and 7 appeared in the electrophoretic patters even at low concentrations of 2-ME, indicating the liberation of these subunits, while B-5 and B-6 did not appear distinctly before the concentration of 2-ME increased to more than 6 mM. As the concentration of 2-ME in-

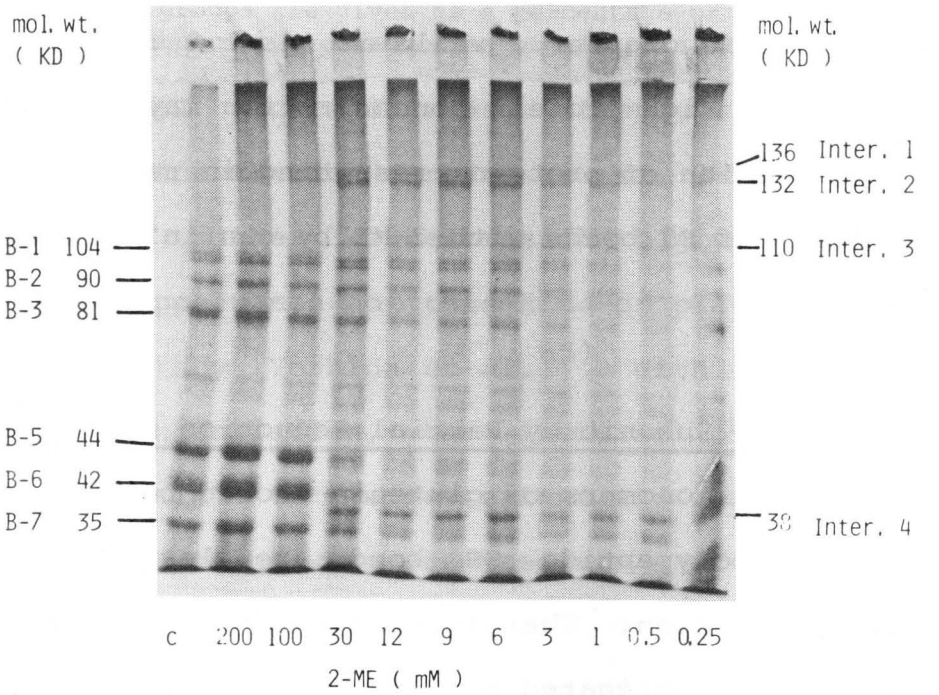


Fig. III-2. SDS-PAGE of Glutenin Partially Reduced with Various Concentrations of 2-ME.

PE-glutenin, obtained by the partial reduction with various concentrations (0.25-200 mM) of 2-ME, was solubilized in SDS buffer of the Laemmli system and submitted to SDS-PAGE. "C" indicates the pattern of the complete reduced PE-glutenin.

creased, all the bands mensioned above increased their intensities except B-4 and 4'. B-4 and 4'appeared even at zero concentration of 2-ME and did not change their intensities with the increase of concentration of 2-ME, showing that these two components are not combined with glutenin polymers by SS linkage and are present in the glutenin fraction as monomers. It was reported in the previous chapter[54] that Cys was scarcely detected in these components as the result of their amino acid analysis.

The band of MW 38 Kd, which was not observed in the complete reduction, appeared at lower concentrations of 2-ME but disappeared at higher concentrations. As reported in detail in chapter IV, the component of MW 38 Kd gave a band in SDS-PAGE at the position of B-6 (MW 42 Kd), when it was separated and reduced completely. In general, when intra-polypeptide SS bonds of proteins are cleaved, their mobilities in gel electrophoresis often decrease because of an expansion of their effective molecular volumes. Therefore, it can be assumed that the component of B-6 appeard at the position of MW 38 Kd with intact intra-polypeptide SS bonds in the trearment with lower concentrations of 2-ME, but was sifted to the position of MW 42 Kd (B-6)

after the reduction at higher 2-ME concentraions. This observations indicates the difference of reacrivity between the inter- and intra-polypeptide SS bonds in the subunit of B-6, suggesting a posibility that the inter-polypeptide SS bonds can be cleaved selectively under appropriate conditions.

Another group of bands were observed in electrophoretic patterns of Fig. III-2 at the positions of MW 136 Kd, 132 Kd , and 110 Kd. These bands appeared at low concentrations of 2-ME, but disappeared at extremely high concentrations. Since their molecular weights were higher than the highest moleculr weight of glutenin subunits, they must be oligomers of glutenin subunits.

In order to examine the liberation of the subunits more exactly , the amount of each subunit liberated by the partial reduction was plotted against the concentration of 2-ME. (results are shown in Fig. III-3). The amount of liberated subunit was determined from the peak area of densitogram in SDS-PAGE and given as a percentage of the area of the same subunit in the complete reduction. As seen in Fig. III-3-B, the liberation of B-7 was more than 90 % even with 1 mM 2-ME. And the liberation of B-7 reached 100 % at about 100 mM 2-ME. On the other hand, of LMW subunit polypeptides, B-6 was not observed distinctly at low concentrations (from 0.5 mM to about 60 mM)

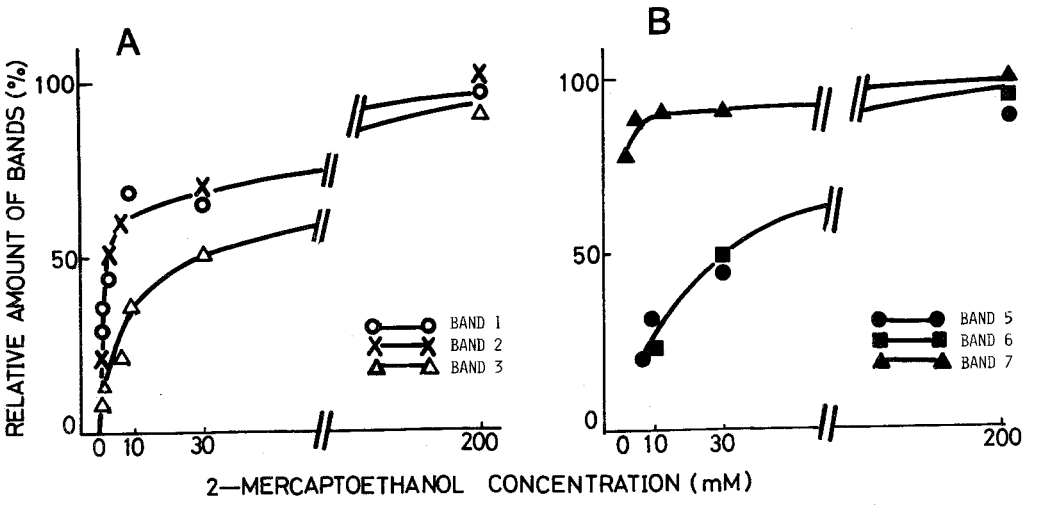


Fig. III-3. Amount of Each Subunit Liberated by Partial Reduction.

The amount of liberated subunit was determined from the peak area of the densitogram in SDS-PAGE (Fig. III-2) and given as a percentage of the area of the same subunits in the complete reduction. Fig. III-3-A, the amounts of B-1, 2 and 3. Fig. III-3-B, the amounts of B-5, 6 and 7.

of 2-ME. But, as described above, B-6 appeared at the position of MW 38 Kd at such low concentraions. Hence, by comparing the area of the MW 38 Kd band with that of B-6 in the complete reduction, it is shown that the liberation of this subunit was also than 90 % at such low concentrations of 2-ME. In contrast to these two components, the amount of the liberation of other glutenin subunits did not increase greatly at low concentrations of 2-ME. B-5 (Fig. III-3-B) was not observed distinctly even with 10 mM 2-ME. Although B-1 + 1', 2, and 3 were liberated at low concentrations of 2-ME (Fig. III-3-A), the increase of liberations according

to the increase of 2-ME concentrations was restricted. Particularlly, the liberation of B-3 was just 50 % even with 30 mM. These results show that inter-polypeptide SS bonds also contained less reactive ones that are not reduced easily with the treatment of low concentrations of 2-ME, in addition to highly reactive ones. That is, the restriction of reactivity with 2-ME can occur not only in intra-polypeptide SS bonds but also in inter-polypeptide ones. The extents of the restriction in inter-polypeptide SS bonds were dependent on the kinds of the subunits. It is thought that this restriction is caused by specific or non-specific subunit-subunit interactions, and that the intensity of such interactions differ according to the kinds of subunits . Thus, the liberation of subunits by reduction seems to reflect subnit-subunit interactions in glutenin.

SH and SS contents of Liberated Subunits.

As described above, the subunit of B-6 (MW 42 Kd, after complete reduction) gave a band at a position of MW 38 Kd in SDS-PAGE, indicating the retention of intra-polypeptide SS bonds at low concentrations of 2-ME. On the other hand, the subunits of B-1, 1', 2, 3, and 7 always appeared at the same positions as those in the

case of complete reduction, independent of the 2-ME concentration. In order to know whether these subunits retain their intra-polypeptide SS bonds at the liberation with a low concentration of 2-ME, the liberated subunits were separated and their SH groups and SS bonds were determined. In this experiment, the reduction of 2-ME was made at a 2-ME concentration of 0.5 mM. After the modification of liberated SH with vinylpyridine, each subunit was separated by SDS-PAGE and then its remaining SS bonds were reduced and alkylated with iodoacetamide. The results of amino acid analysis with the subunit polypeptides thus obtained are shown in Table III-I. In this analysis, the amounts of SH group and SS bond in the liberated subunits were given as amounts of PE-Cys and CM-Cys, respectively. In other words, the amounts of PE- and CM-Cys represent that of inter- and intra-polypeptide SS bonds existing in separated subunits, respectively.

According to the results reported in the previous chapter,[54] each of the glutenin subunits separated by SDS-PAGE contained about 4 mol Cys per mol protein. If glutenin subunits are linked linearly, each subunit liberated by reduction should have 2 mol SH (corresponding to the inter-polypeptide SS bonds) per mol protein.

Table III-I

Amino Acid Composition of Subunit Polypeptides of PE-, CM-Glutenin*

	B-1	B-2	B-3	B-7
Lys	30.0	26.2	26.6	5.4
His	16.0	16,4	16.5	5.8
Arg	40.1	35.0	32.8	9.4
Asp	60.2	54.2	50.3	11.1
Thr	31.9	31.5	30.5	15.0
Ser	99.4	75.9	55.9	24.3
Glu	165.6	152.9	128.2	92.5
Pro	58.1	43.0	51.5	37.4
Gly	179.9	132.8	93.2	21.2
Ala	44.2	47.7	53.2	13.1
Val	37.1	36.3	38.1	16.0
Met	9.8	9.9	10.6	4.6
Ile	29.0	27.3	27.1	12.5
Leu	60.6	54.5	53.1	23.4
Tyr	29.0	31.8	21.9	5.2
Phe	23.7	22.2	22.4	12.2
**PE-Cys	0.6	tr	tr	0.3
CM-Cys	4.4	3.4	4.0	3.8

*Values shown are expressed as residue number per mol protein.
**Amounts of PE- and CM-Cys represent SH and SS in the liberated subunits, respectively.

If subunits are linked with some branching here and there, the amount of SH should be more than 2 mol. However, the amount of PE-Cys shown in Table III-I was much less than 2 mol per mol protein, and most of the Cys was detected as CM-Cys. This result indicates that liberated subunits did not have SH groups originated from inter-polypeptide SS bonds, but have only intra-polypeptide ones. Thus, a simple explanation for this results is that glutenin is not a polymer of polypeptides linked through SS bonds, but exist as monomers.

This kind of structure has been proposed by a few authors, [1,30,34] but lacked reliable experimental evidence. On the other hand, there is much evidence that glutenin is a polymer consisting of polypeptides linked through SS bonds. Glutenin behaves as a high MW polymer in concentrated urea or guanidine hydrochloride solutions, [29,6] and their MW decrease by cleavage of SS bonds .[1] Neverthless, the result of Table III-I, as described above, does not support the polymer structure of glutenin. Therefore, the reason why the separated subunits gave such low values of PE-Cys and high values of CM-Cys in spite of the liberation of subunits through reductive cleavage of SS bonds must be considered.

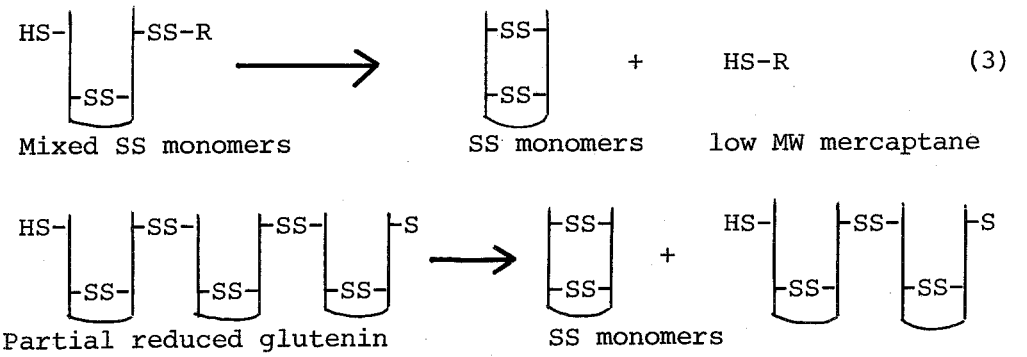
The unexpectidly low amounts of PE-Cys found in the liberated subunits may be explained partly by the production of mixed SS on cleavage of interpolypeptide SS bonds. Cleavage of SS bonds through SH-SS exchange reaction occurs in the following two steps :

$$\text{P-SS-P} + \text{R-SH} \longrightarrow \text{P-SS-R} + \text{SH-P} \qquad (1)$$

$$\text{P-SS-R} + \text{R-SH} \longrightarrow \text{P-SH} + \text{R-SS-R} \qquad (2)$$

while one SS bond give two SH in the presence of excess reducing agent, the reaction stops at the first stage when the amount of the reducing agent is insufficient.

This leads to the formation of mixed SS. In the experiment described above, cleavage of SS bonds was made with a low concentration of 2-ME (0.5 mM). This concentration would be 25 folds of the concentration of glutenin SS bonds, if the glutenin was homogeneous. Actually, some precipitates of glutenin appeared in the reaction mixture on adjusting the pH to 6.0. Since the concentration of 2-ME seems to be very low in the precipitate, the concentration of glutenin SS bonds should exceed that of 2-ME, resulting in the formation of mixed SS. However, cleavage of one SS bond should give one SH at least as shown in (1), (2). Hence, the low values of PE-Cys obtained in Table III-I can not be explained only by the formation of mixed SS. In order to explain the low amount of PE-Cys in the liberated subunits, the following inter-polypeptide interchange reactions must be assumed.



With this kind of reaction, monomer subunits having no SH can be liberated as illustrated above in (3) or (4). The low concentration of the reducing agent to that of protein SS bonds favors the formation of this type of subunit monomer . It was reported that this type of subunit monomer was obtained when complete reduced glutenin was reoxidized.[31-,67] Therefore, in the present experiments, it can be expected that this kind of reaction would occur. Further, SH groups liberated by partial reduction might be reoxidized before they were pyridylethylated, since pyridylethylation of SH groups might not proceed easily owing to the precipitate.

CHAPTER IV

Selective Reduction of Inter- and Intra-Polypeptide Disulfide Bonds of Wheat Glutenin.

IV-1 Introduction

Wheat glutenin is thought to be a polymer protein which consists of several kinds of subunit polypeptides linking together through SS bonds.[20,21] Such a structure of glutenin molecule is considered to be a main cause for the unique viscoelastic property of wheat flour dough.[67,68]

However, it has not been elucidated clearly how many SS bonds are present in each glutenin subunit and how each subunit links to adjacent subunits through SS bonds. Simply, two structural models of glutenin molecule can be proposed as illustrated in Fig. IV-1; in case that each subunit has two inter-polypeptide SS bonds, glutenin may compose a linear structure like Fig. IV-1-A.[40] If each subunit has inter-polypeptide SS bonds more than three, glutenin may take three dimensional network structure through SS bonds like Fig. IV-1-B.[22,36]

In the previous chapters,[54,66] in order to determine the number of inter- and intra-polypeptide SS

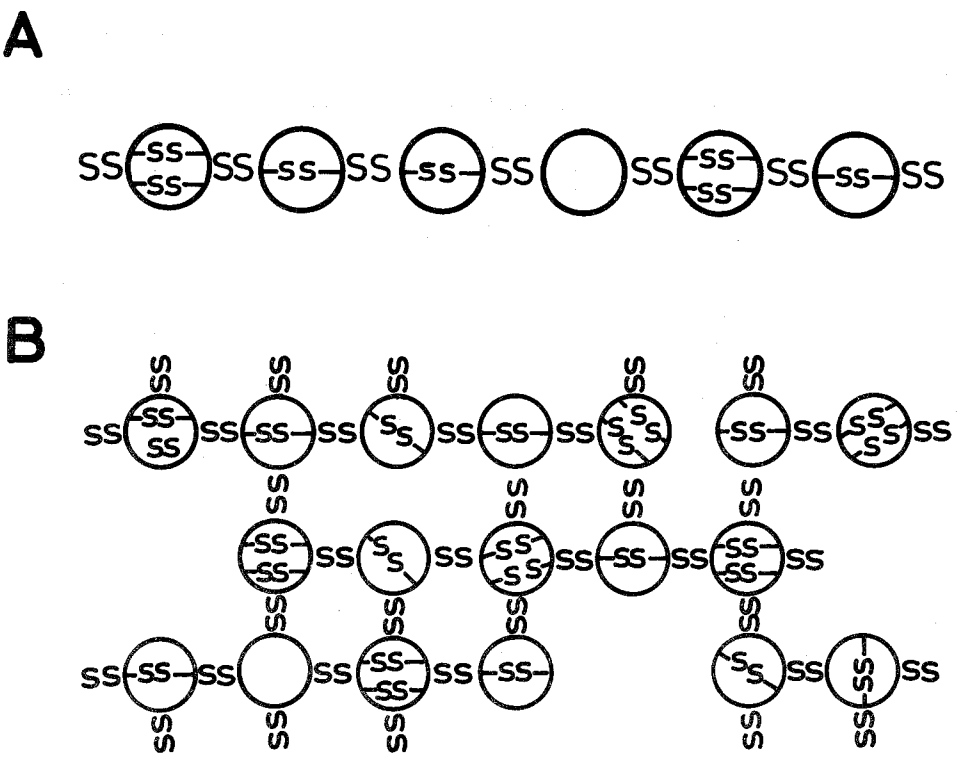


Fig. IV-1. Two Structural Models of Glutenin Molecule.

(A) The linear structure, in which each subunit has two inter-polypeptide SS bonds. (B) The three dimensional net work structure, in which each subunit has more than three inter-polypeptide SS bonds.

bonds of each subunit, we first isolated main glutenin subunits and determined cysteine content per each subunit.[54] Further, we tried to find the condition of selective cleavage of only inter-polypeptide SS bonds by reducing glutenin with various concentrations of 2-ME at pH 6.0.[66] But we could not detect SH groups which seemed to be involved in inter-polypeptide SS bonds. This may be explained by assumption that SH

groups liberated in protein seems to be easy to reform SS bonds again, since glutenin aggregated rapidly at pH 6.0 and probably the concentration of 2-ME was fairly low in the precipitate of glutenin, and that alkylation of SH groups might be imcomplete owing to the precipitate, resulting in low value of SH content. Thus, in the present chapter,[70] in order to exclude the effect of precipitation, reduction of glutenin and alkylation of SH groups liberated was carried out in solution at pH 4.0. This condition enabled us to cleave and modify inter- and intra-polypeptide SS bonds selectively. Then, the number of inter- and intra-polypeptide SS bonds of each subunit was determined.

IV-II MATERIALS AND METHODS

Materials Wheat flour (60 % extract of NO. 1 CW wheat milled by a test mill) was provided by Nisshin Flour Milling Co. Ltd. Kobe. Iodoacetamide (IAM) was purchased from the Protein Research Foundation. Other reagents were obtained from Wako Pure Chemical Ind. Ltd. or Nakarai Chemicals Ltd.

Preparation of Glutenin.

Glutenin was prepared from wheat flour according

to the method as described in chapter II-2.

Reduction and Alkylation.

The partial reduction of SS bonds of glutenin and carboxymethylation of SH groups was performed as follows. Glutenin was dissolved completely in 0.01 M acetic acid in a protein concentration of 0.04 % and adjusted pH to 4.0. The glutenin solution was deaired by N_2 gas and 2-ME was added to the solution in various concentrations. After reduction at 20°C for 4 hr, twice molar excess of IAM compaired with SH groups originated from glutenin and 2-ME, was added and alkylation (carboxymethylation) was carried out at 20°C for 16 hr at pH 4.0. During the process of carboxymethylation, I^- was liberated and pH of solution falled gradually, particularly in the solution containing high concentration of 2-ME and IAM. Hence, pH was sometimes adjusted to 4.0 with 0.1 N NaOH. In spite of carboxymethylation for 16 hr, there was possibility that some of SH groups of glutenin and/or of 2-ME remained unmodified. So it was necessary to add the denaturant to the solution and raise the pH of the solution in order to modify SH groups in glutenin completely. However, addition of denaturant and raising of pH may further

cause the cleavage of SS bonds of glutenin owing to unmodified 2-ME. Thus, first, in order to remove unmodified 2-ME, the reaction mixture was dialyzed against 0.1 M acetic acid under N_2 bubbling. Next, in order to modify the unreacted SH groups in protein completely, the reaction mixture was dialyzed against 0.2 M Tris-HCl buffer, pH 8.0, containing 8 M urea, 1 mM EDTA, 10 mM IAM for 16 hr. Then the reaction mixture was dialyzed against 0.1 M acetic acid to remove the reagents and lyophilized. Partially-reduced and carboxymethylated glutenin (CM-glutenin) was thus obtained.

Complete reduction and pyridylethylation of SH groups was carried out as follows; Glutenin was dissolved in 0.3 M Tris-HCl buffer (pH8.0), containing 8 M urea, 1 mM EDTA in a protein concentration of 1 %. The glutenin solution was deaired by N_2 gas and 5 mM of 2-ME was added. After reduction at 20°C for 4 hr, 10 mM of vinylpyridine was added. Pyridylethylation of SH groups was carried out at 20°C for 16 hr. The reaction mixture was dialyzed against 0.1 M acetic acid and lyophilized.

SH Determination by Amino Acid Analysis.

S-Alkylated glutenin and glutenin subunits were

hydrolyzed with 6 N HCl in a sealed and evacuated tube at 110°C for 22 hr. Cysteine residues in the hydrolyzate were determined as S-carboxymethyl cysteine (CM-Cys) and S-pyridylethyl cysteine (PE-Cys) by amino acid analyzer (Hitachi 835).

SDS-PAGE

SDS-PAGE was carried out using the discontinuous buffer system of Laemmli.[59] In the case of analytical SDS-PAGE, electrophoresis was performed on a slab gel (1 X 160 X 120, mm), at 20 mA / slab, for 4-5 hr. In the case of preparative one, electrophoresis was performed on a slab gel (5 X 210 X 150, mm), at 20 mA / slab, for 16 - 20 hr. After being stained with 0.1 % Coomassie brilliant blue R-250 in a methanol-water-acetic acid solution (50 : 10 : 40, V/V) for 20 min, the gel was destained in a methanol-water-acetic acid solution (10 : 83 : 7, V/V) with continious shaking. The gel of analytical SDS-PAGE was dried on a filter paper. The gel of preparative SDS-PAGE was used for the electric extraction of protein, which will be described in the following section.

Electric Extraction of Subunit Polypeptides from Gels of Preparative SDS-PAGE.

The main bands in preparative SDS-PAGE gel were cut out with a razor blade and crushed to small pieces. The crushed gels were stirred in 0.125 M Tris-HCl buffer pH 6.8 containing 4 % SDS and 20 % glycerol for 12 hr and heated at 100°C. The electric extraction of protein from the gel was carried out with a glass tube (20 mm diameter) according to the method of electrophoretic elution.[60] The extraction was performed at 40 mA for 16-20 hr at 20°C using the buffer system of Laemmli.[59] The solution containing extracted subunit polypeptides was dialyzed against 0.1 M acetic acid and lyophilized. SDS binding to subunit polypeptids was removed according to the method of Henderson.[71]

IV-3 RESULTS AND DISCUSSION

Liberation of SH Groups from Glutenin by Partial Reduction.

Glutenin was dissolved in 0.01 M acetate buffer , pH 4.0, and reduced with various concentrations of 2-ME. SH groups liberated were carboxymethylated with IAM. CM-Cys in CM-glutenin was determined by amino acid analysis after 6 N HCl hydrolysis. Fig. IV-2 shows the

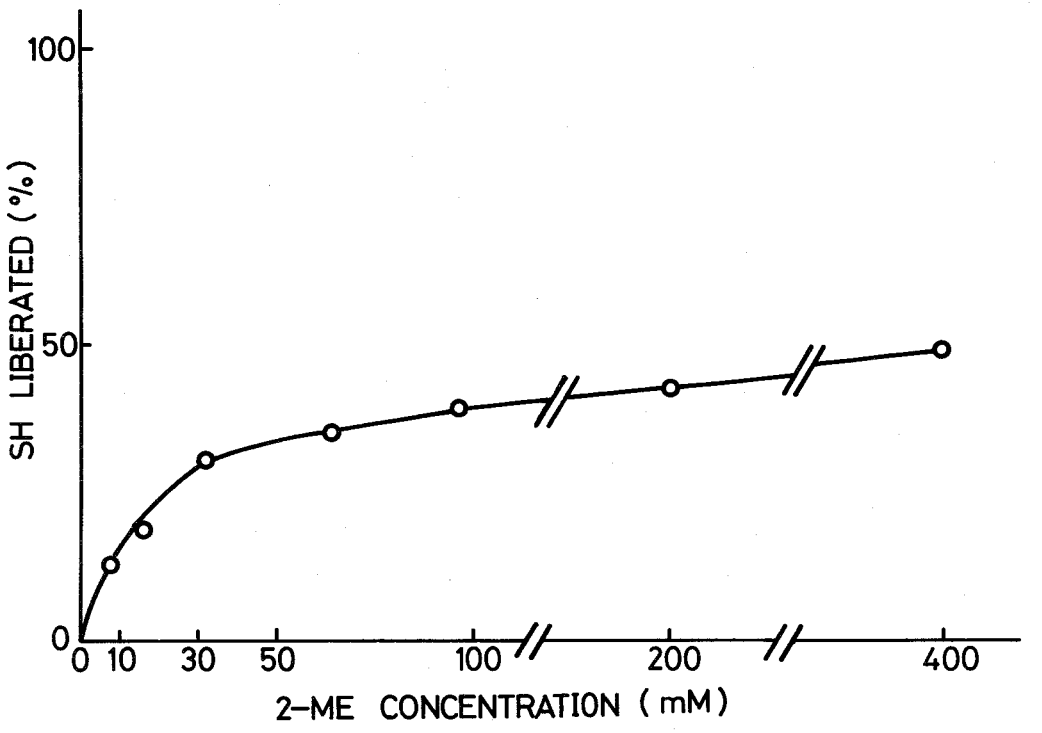


Fig. IV-2. Liberation of SH Groups from Glutenin by Partial Reduction with Various Concentrations of 2-ME.

SH groups liberated by the partial reduction was carboxymethylated with IAM and determined as CM-Cys by amino acid analysis. Amount of SH groups are given as a percentage of the total SH groups liberated from glutenin by complete reduction.

liberation of SH groups in glutenin as a function of the concentration of 2-ME. The amount of SH groups liberated first increased steeply with the increase of 2-ME concentration to 32 mM and reached to about 30 % of total SH groups at 32 mM. At the concentration of 2-ME above 32 mM, the liberation of SH groups was very low and attained only about 50 % of total SH groups in 400 mM 2-ME.

The fact that only 50 % of total SS bonds was susceptible to a high concentration of 2-ME such as 400 mM indicates that reduction occurred under the relatively mild conditions, since reactivity of 2-ME was fairly low at pH 4.0 where the conformation of glutenin subunits was still compact. Among SS bonds cleaved under this condition, about 60 % (30 % of total SS bonds) which was easily cleaved with 2-ME below 32 mM seemed to be high reactive ones. Similar results were also reported by Ewart [35] that 40 % of total SS bonds were highly susceptible to reducing agents.

Liberation of Glutenin Subunits by Partial Reduction

If SS bonds which are easily cleaved below 32 mM correspond to inter-polypeptide SS bonds, some subunits with uncleaved intra-polypeptide SS bonds should be liberated. To examine this possibility, the mode of liberation of subunits by partial reduction with 2-ME was analyzed by SDS-PAGE. Fig. IV-3 shows the electrophoretic profiles. Lane c is the pattern of the completely reduced and carboxymethylated glutenin. The bands at the position of molecular weight = 104, 91, 81, 60, 44, 42 and 35 Kd, represent glutenin subunits and are designated as B-1, B-2, B-3, B-4, B-5, B-6 and

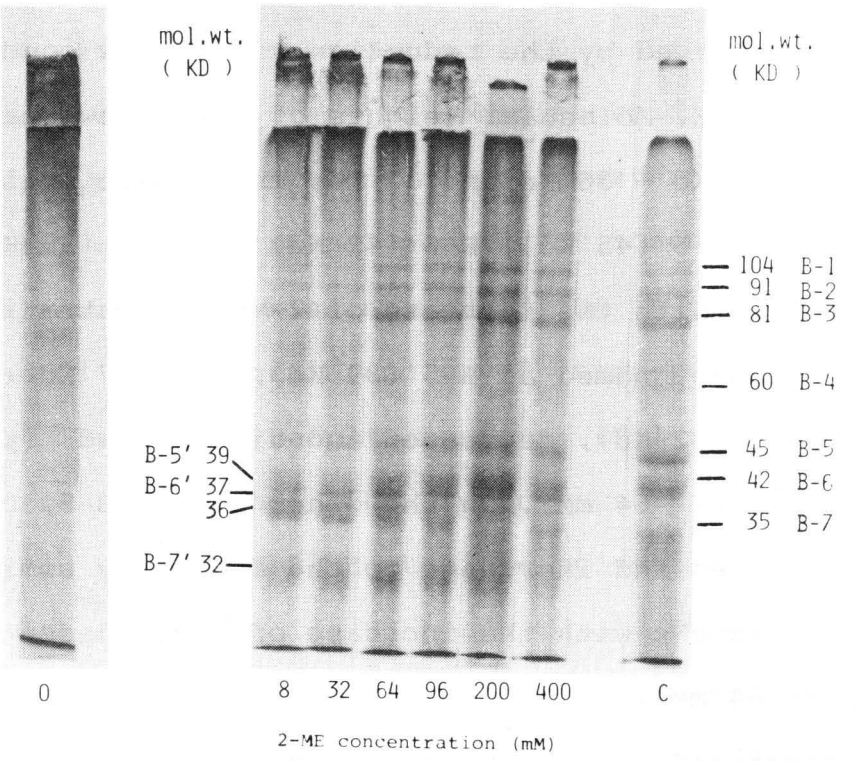


Fig. IV-3. SDS-PAGE of Glutenin Partially Reduced with Various Concentrations of 2-ME.

CM-glutenin, obtained by the partial reduction with various concentrations (8-400 mM) of 2-ME, was solubilized in SDS buffer of the Laemmli system and applied to SDS-PAGE without 2-ME. "C" indicates the pattern of the completely reduced CM-glutenin. "0" 2-ME sample was subjected to the same condition as the others but in the absence of 2-ME.

B-7, respectively. When profiles of glutenin reduced with 8-400 mM 2-ME were compared with that of completely reduced glutenin, high molecular weight bands (HMW bands), that is B-1, 2, and 3, were liberated at a low concentration of 2-ME and increased with the increase of 2-ME concentration. Several low molecular weight bands (LMW bands) other than main subunits (B-5, 6, 7)

were newly observed by the reduction with a low concentration of 2-ME. Molecular weights of these new bands were 39 Kd, 37 Kd + 36 Kd and 32 Kd, which were smaller than those of B-5 (45 Kd), B-6 (42 Kd) and B-7 (35 Kd), respectively. With the increase of 2-ME concentration, these new bands, named as B-5'(39 Kd), B-6'(37 Kd + 36 Kd) and B-7' (32 Kd), decreased and disappeared (particularly above 64 mM). On the other hand , B-5, B-6 and B-7 appeared and increased to the intensity similar to that in lane c with the increase of 2-ME concentration (above 64 mM).

As suggested in the preceding chapter,[66] the Author interpreted the appearance of B-5', 6' and 7' at a low concentration of 2-ME and the shift of these bands to B-5, 6 and 7, respectively, with the increase of 2-ME concentration as follows: B-5', 6' and 7', which were liberated by the partial reduction of glutenin with a low concentration of 2-ME such as 8-32 mM at acidic pH, must retain intra-polypeptide SS bonds though the inter-polypeptide SS bonds of them were cleaved. With the increase of 2-ME above 64 mM, not only inter-polypeptide SS bonds but also intra-polypeptide SS bonds were cleaved. Intra-polypeptide SS bonds may compose the compact conformation of glutenin

subunit even in the presence of SDS. Hence, the electrophoretic mobilities of subunits holding uncleaved intra-polypeptide SS bonds may be greater than the same subunits whose intra-polypeptide SS bonds have already been cleaved. If this interpretation is correct, B-5', 6' and 7' must contain uncleaved intra-polypeptide SS bonds. From the results in Figs. IV-2 and IV-3, we considered that under the present conditions, partial reduction by 32 mM 2-ME attained selective cleavage of only inter-polypeptide SS bonds of glutenin, since at 32 mM 2-ME, only B-5', 6' and 7' were liberated with the trace amount of B-5, 6 and 7, but above 64 mM 2-ME, B-5' 6' and 7' began to decrease with the increase in B-5, 6 and 7.

Separation of Glutenin Subunits Retaining Intra-Polypeptide SS Bonds

Glutenin subunits retaining intra-polypeptide SS bonds were separated according to the procedure shown in Fig. IV-4. Glutenin was partially reduced with 32 mM 2-ME under the same condition as that in the above section. After carboxymethylation of SH groups liberated, CM-glutenin was applied to a preparative SDS-PAGE to separate subunits. Each band of subunit (B-1, 2, 3,

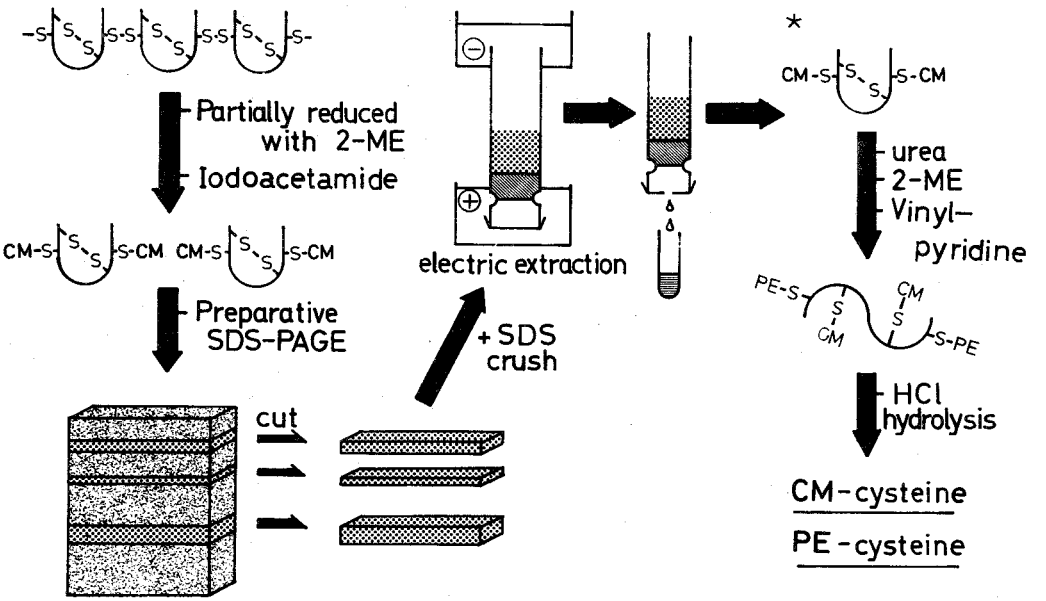


Fig. IV-4. The Procedure for Separation of Subunit Polypeptides Retaining Intra-polypeptide SS Bonds and Determination of Inter- and Intra-polypeptide SS Bonds of Each Subunit.

Main subunit polypeptides (B-1, 2, 3, 5', 6' and 7') were separated from glutenin which was partially reduced with 32 mM 2-ME. Separated subunit polypeptides are represented with asterisk "*".

5', 6' and 7') was cut out from the preparative gel , and subunit polypeptides were extracted from the gels by electric extraction. The homogeneity of each extracted polypeptide (they are represented with asterisk * in Fig. 4) was checked by SDS-PAGE.

HMW-bands, B-1, 2 and 3 were well separated as shown in Fig. IV-5 (- 2-ME). These bands didn't change their mobilities on SDS-PAGE after complete reduction (Fig. IV-5, + 2-ME). The separation of LMW-bands (B-5',

6' and 7') was well attained as shown in Fig. 6(-2-ME). By complete reduction, band 5', 6' and 7' decreased their mobilities and shifted to the positions of B-5, 6 and 7, respectively (Fig. IV-6, + 2-ME). These results support our previous interpretation that B-5', 6' and 7' has uncleaved intra-polypeptide SS bonds and are the same subunits as B-5, 6 and 7, respectively. In contrast to LMW-subunits, HMW-subunits,

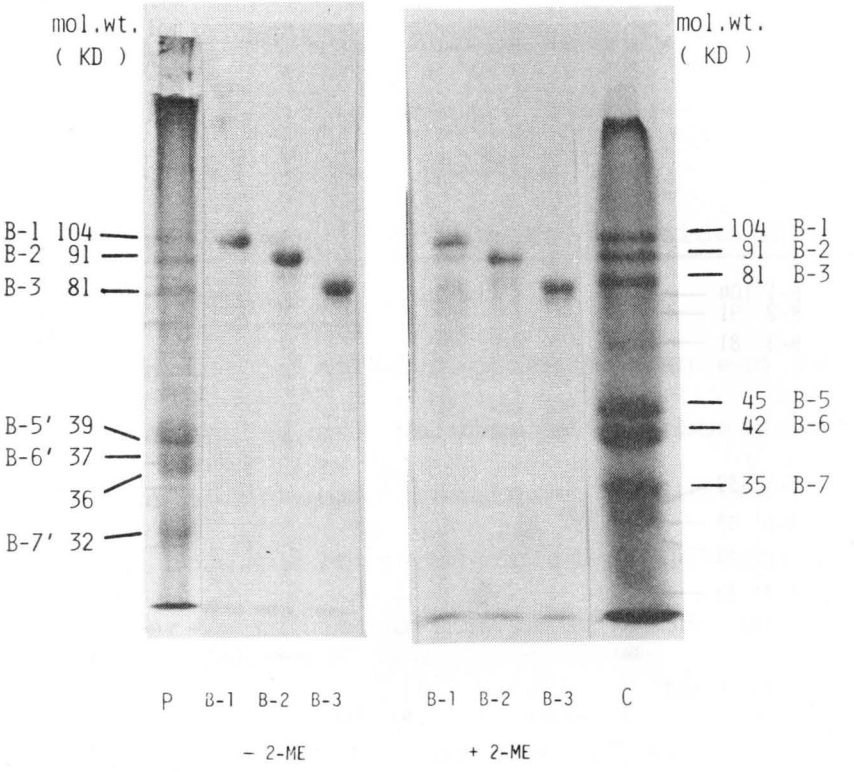


Fig. IV-5. SDS-PAGE of Separated HMW Bands.

Separated HMW bands (B-1, 2 and 3) subunit polypeptides were solubilized in SDS buffer of the Laemmli system and applied to SDS-PAGE. + 2-ME and -2-ME indicate the patterns of separated subunits in the presence and absence of 2-ME, respectively. "P" and "C" indicate the patterns of partially reduced (with 32 mM 2-ME) CM-glutenin and completely reduced CM-glutenin, respectively.

B-1, 2 and 3 did not change their mobilities after complete reduction. We consider this as follows : B-1, 2 and 3 liberated at 32 mM 2-ME contain intra-polypeptide SS bonds, but the contribution of intra-polypeptide SS bonds to their conformation is not so great as that in LMW-subunits. Accordingly the shape of SDS-(HMW-subunits) complex must be similar between subunits with and without intra-polypeptide SS bonds,

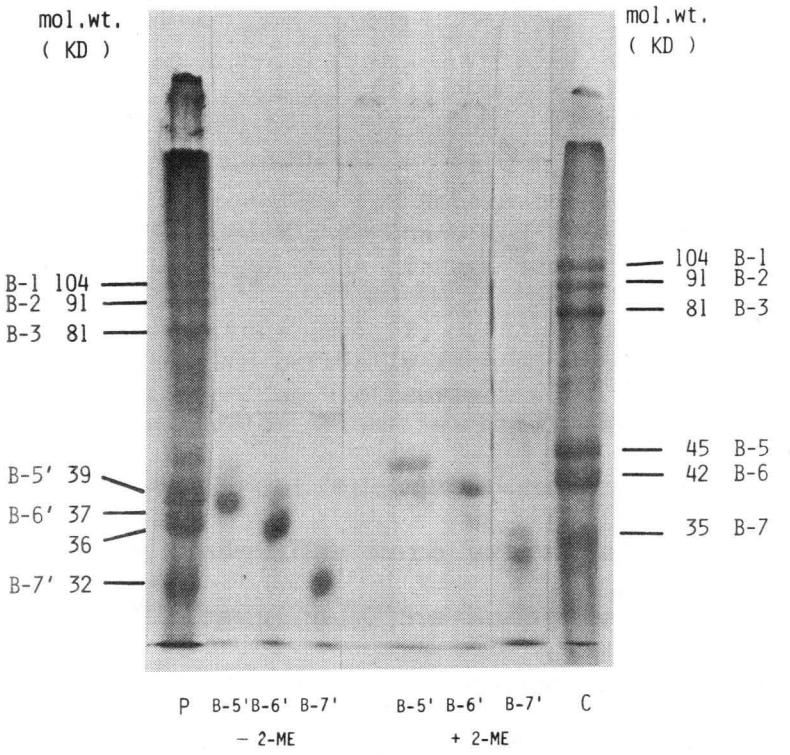


Fig. IV-6. SDS-PAGE of Separated LMW Bands.

Separated LMW bands (B-5', 6' and 7') subunit polypeptides were solubilized in SDS buffer of the Laemlli system and applied to SDS-PAGE. + 2-ME and - 2-ME indicate the patterns of separated subunits in the presence and absence of 2-ME, respectively. "P" and "C" indicates the patterns of partially reduced (with 32 mM 2-ME) CM-glutenin and completely reduced CM-glutenin, respectively.

resulting in the same elctrophoretic mobilities regardless of the absence or presence of 2-ME. A small amount of bands other than the subunit bands was observed in the presence of 2-ME (Figs. IV-5 and 6 (+ 2-ME)). This indicates that each band liberated by partial reduction contained a small amount of other subunits polymerized through SS bonds. However, densitometric determination showed that the content of the other contaminated subunits were usually below 5 % (data not shown).

Determination of Inter- and Intra-Polypeptide SS Bonds in Each Subunit Polypeptides

The results in the above section guaranteed us the selective cleavage of inter-polypeptide SS bonds and the good separation of subunits retaining uncleaved intra-polypeptide SS bonds. Thus, we tried to determine the number of inter- and intra-polypeptide SS bonds in separated subunit polypeptides according to the procedure shown in the latter part of Fig. IV-4.

Separated subunits, B-1, 2, 3, 5', 6' and 7', (they are represented with asterisk * in Fig. IV-4) were completely reduced with 2-ME in the presence of 8 M urea at pH 8.0 and SH groups liberated were pyridyl-

Table IV-I

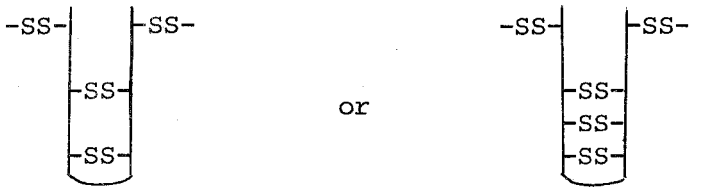
Amino Acid Compositions of Subunit Polypeptides of CM-, PE-Glutenin[a]

	B-1	B-2	B-3	B-5'	B-6'	B-7'
Lys	9.3	9.2	7.1	4.6	4.4	3.7
His	10.1	9.9	11.2	4.4	4.5	4.2
Arg	19.1	16.0	13.3	8.3	7.7	7.3
Asp	17.1	18.0	13.3	8.3	7.7	7.3
Thr	25.4	22.7	19.1	9.7	8.7	8.0
Ser	64.7	66.3	51.9	28.4	26.0	17.9
Glu	253.9	208.8	205.9	108.2	100.1	76.3
Pro	112.0	87.5	53.7	55.6	50.7	43.7
Gly	134.8	112.1	93.5	23.1	20.3	14.3
Ala	31.4	28.3	23.0	11.7	12.0	9.1
Val	25.3	20.7	19.7	17.6	17.5	14.2
Met	3.7	2.5	2.8	3.1	3.7	3.2
Ile	17.9	15.6	14.4	13.2	14.6	12.9
Leu	47.3	35.8	34.7	30.6	27.8	22.2
Tyr	33.2	28.5	23.5	6.1	6.2	6.4
Phe	17.4	15.4	15.2	12.4	11.4	9.8
CM-Cys[b]	1.8	1.6	1.9	1.7	2.2	2.0
PE-Cys	7.0	4.7	4.3	4.2	4.7	5.1

[a]Values are expressed as residual numbers per mole protein. Values are the averages of duplicate. Standard error is within 5 %.
[b]CM- and PE-Cys represent SH groups involved in inter- and intra-polypeptide SS bond, respectively.

ethylated with vinylpyridine followed by 6 N HCl hydrolysis to determine CM-Cys and PE-Cys by amino acid analysis. Thus, in this procedure, CM-Cys and PE-Cys represent cysteine residues involved in SS bonds of inter- and intra-polypeptide , respepctively. Amino acid compositions of each subunit polypeptides is shown in Table I. Values are given as amino acid residual number per mole subunit protein. Concerning CM-Cys contents, all subunits had 2 moles CM-Cys per mole subunit. In contrast, concerning PE-Cys, B-1 had 7.0 moles PE-Cys and other bands 4-5 moles PE-Cys per mole subunit. Glutenin used in the present study had no free SH groups (data not shown). Hence, the number of

CM-Cys and PE-Cys in Table I indicates that each subunit contains 2 inter-polypeptide SS bonds in common and 2-3 intra-polypeptide SS bonds as shown below.



The results described above clearly demonstrate that glutenin was a polymer of several subunit polypeptides linked "linearly" through 2 inter-polypeptide SS bonds as shown in Fig. IV-1-A. As far as intra-polypeptide SS bonds, each subunit was shown to have 2-3 intra-polypeptide SS bonds as follows: 3.5 for B-1, 2.5 for B-2, 6' and 7', and 2.0 for B-3 and 5'. Each subunit should have the integral number of intra-polypeptide SS bonds since no free SH groups are present in glutenin. Nevertheless, non-integral numbers such as 3.5 and 2.5 were obtained in the separated subunits. There are two possible explanations for such non-integral numbers : first, the bands (subunits) separated from SDS-PAGE may contain polypeptides with different numbers of intra-polypeptide SS bonds, though their molecular weights (mobility on SDS-PAGE) are the same. Second, fractions of separated subunits, especially HMW-subunits, may contain not only monomer subunits but

also dimer or trimer which are consisted of other LMW subunits linked through SS bonds as observed in Figs. 5 and 6, since partial reduction may produce dimer or trimer. For example, if we take the assumption that 90 % of B-2 fraction (91 Kd) are monomers with 2 moles intra-polypeptide SS bonds and 10 % are dimers, B-2 would have 2.3 moles intra-polypeptide SS bonds per subunit on the average by the following equation:

Average numbers of intra-polypeptide SS bonds of B-2

$$= \frac{2 \text{ (moles)} \times 90(\%) + 5 \text{ (moles)} \times 10(\%)}{100 \ (\%)} = 2.3 \text{ moles}$$

The present study demonstrates that glutenin consists of several kinds of subunits which linked linearly through inter-polypeptide SS bonds. However, this does not always imply glutenin molecule takes a linear stretched form in a hydrated state or in solution. Glutenin subunits are thought to interact with another ones in the same molecule strongly through non-covalent interaction (namely, hydrophobic interaction and hydrogen and ionic bonds etc.) and associate densely here and there in glutenin molecule. Further, such association is thought to occur also between glutenin molecules. Owing to such intra- and inter-molecular associations which may be a main cause for visco-

elasticity of glutenin, glutenin is considered to take the complicated three dimensional structure. The mode of association between glutenin subunits or between glutenin molecules is thought to be governed by the nature of subunits. The present study offered the condition of selective cleavage of inter-polypeptide SS bonds. Thus, we are now trying to separate glutenin subunits in a native state in the absence of SDS or other denaturant, which enable us to study the nature of glutenin molecule.

Chapter V

Interaction Between Subunit Polypeptides of Glutenin through Non-Covalent Forces

V-1 INTRODUCTION

In the previous chapter,[70] it was elucidated that glutenin is a polymer of several kinds of subunit polypeptides linked "linearly" through two inter-polypeptide SS bonds as shown in Fig. IV-1-A. However, this does not always mean glutenin molecule takes a "linear stretched form". Since Glutamine and hydrophobic amino acid contents are very high in glutenin,[72,73] each glutenin subunit seems to interact with another ones through non-covalent forces (namely, hydrophobic interaction and hydrogen and ionic bonds etc.). Because of such subunit-subunit interaction through non-covalent forces, glutenin subunits are thought to associate densely here and there in one glutenin molecule. Further, such subunit-subunit interaction seems to occur also between glutenin molecules. Owing to such intra- and inter-molecular associations between glutenin subunits, glutenin is considered to take the complicated three dimensional structure. There were some reports[6,53,64,74] inves-

tigating subunit-subunit interaction through non-covalent forces by carrying out the gel filtration of reduced, alkylated glutenin in various solutions. These reports showed that a part of subunit polypeptides of glutenin associated in acetic acid, urea and SDS solution and formed the aggregative fraction (F-I). Although these reports also showed the subunit polypeptide compositions of F-I fraction, it was not elucidated yet what kinds of subunit polypeptides were really aggregative. Thus, in the present chapter, in order to identify the really-aggregative subunit polypeptides and investigate how such aggregative subunits interact with other subunits through non-covalent forces, gel filtrations of CE- and PE-glutenin on Sephadex G-150 were performed in the presence or absence of 8 M urea.

V-2 MATERIALS AND METHODS

Materials.

Wheat flour (60 % extract of No. 1 CW Wheat milled by a test mill) was provided by the Nisshin Flour Milling Co., Ltd., Kobe. Sephadex G-150 was purchased from Pharmacia Fine Chemicals, Uppsula, Sweden. Other reagents were obtained from Wako Pure Chemical Ind., Ltd. or Nakarai Chemicals Ltd.

Preparation of Glutenin.

Glutenin was prepared from wheat flour according to the same method as described in chapter II-2.

Reduction and Alkylation of Glutenin.

Reduction of SS bonds of glutenin and pyridylethylation of SH groups were carried out according to the methods of Friedman et al.[58] Reduction of SS bonds and cyanoethylation of SH groups were carried out according to the methods of Arakawa et al.[75]

Gel Filtration Chromatography of CE- and PE-glutenin.

Sephadex G-150 gel chromatography was performed using the column (5.0 x 100, 2.5 x 100, 1.5 x 90 or 1.25 x 100 cm) equilibrated with 0.1 M acetic acid or 0.1 M acetic acid - 8 M urea. After CE-glutenin was dissolved in 0.1 M acetic acid or 0.1 M acetic acid - 8 M urea and PE-glutenin was dissolved in 0.1 M acetic acid, they were applied on the columns. Absorbance at 280 nm was determined for each fraction with a Shimadzu UV-visible recording spectrophotometer UV-260.

SDS-PAGE.

SDS-PAGE, and staining and destaining of the gel were carried out as described in the section of Ana-

lytical SDS-PAGE in chapter II-2.

V-3 RESULTS AND DISCUSSION

Gel Filtration of CE- or PE-glutenin on Sephadex G-150.

Results of gel filtration of CE- and PE-gluenin on Sephadex G-150 are shown in Fig. V-1. Fig. V-1-A shows the elution profile obtained when subunit polypeptides of CE-glutenin were fractionated by using 0.1 M acetic acid as an eluant. This profile reveals that subunit polypeptides of CE-glutenin were fractionated into three fractions, F-I, F-II and F-III. There have been several publications concerning that gel filtration of reduced alkylated glutenin in acetic acid, urea or SDS solutions produced such three peaks, indicating that F-I fraction eluting in void volume contained aggregates of subunit polypeptides.[6,63,5373] Since subunit polypeptides of glutenin contained a high content of glutamine, hydrogen bond is thought to be responsible for such aggregation of subunit polypeptides in F-I fraction. Thus, gel filtration of CE-glutenin was performed in the presence of urea, which seems to weaken primarily hydrogen bond (Fig. V-1-B). The eluant used was 0.1 M acetic acid - 8 M urea. The peak of F-I fraction disappeared and only two peaks of F-II

and F-III were observed. This indicates that 8 M urea dissociated the aggregation of subunit polypeptides in F-I fraction completely. On the other hand, Khan and Bushuk[73] reported that the aggregation of subunit polypeptides in F-I fraction could not be dissociated completely by 0.1 % SDS, which disrupts hydrophobic interaction in solution. Thus, the aggregation of the subunit polypeptides in F-I fraction may be due to hydrogen bond mainly. In order to clarify the effect of ionic bond on the aggregation of subunit polypeptides, gel filtration of PE-gltenin was performed by using 0.1 M acetic acid as a eluant (Fig. Vl-C). Plus charge of PE-glutenin was increased by the addition of pyridylethyl group to glutenin, compared with CE-glutenin which had the same net charge as glutenin. The elution profile of PE-glutenin (Fig. V-l-C) showed that the positon of F-I fraction peak shifted slightly to the side of low molecular weight and the amount of this fraction also decreased, compared with those of CE-glutenin (Fig. V-l-A). This suggests that the increase of plus charge weakened the aggregation of subunit polypeptides in F-I fraction. Hence, ionic bond also seems to contribute to the aggregation of subunit polypeptides of glutenin. From the results shown in Fig.

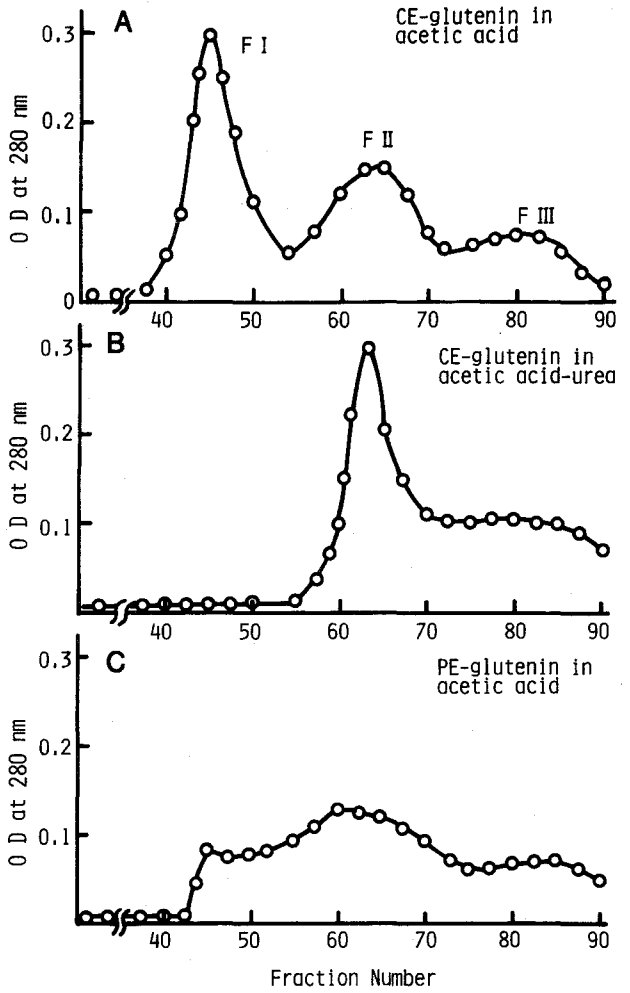


Fig. V-1. Gel Filtration of CE- PE-Glutenin on Sephadex G-150.

(A) CE-glutenin (200 mg) was dissolved in 0.1 M acetic acid (10 ml) and applied on a Sephadex G-150 column (5.0 x 100 cm) equilibrated with 0.1 M acetic acid. Elution was performed with 0.1 M acetic acid. Fractions of 18 ml were collected and absorbance at 280 nm was determined for each fraction.
(B) CE-glutenin (100 mg) was dissolved in 0.1 M acetic acid - 8M urea (5 ml) and applied on a Sephadex G-150 column (2.5 x 100 cm) equilibrated with 0.1 M acetic acid - 8 M urea. Elution was performed with 0.1 M acetic acid - 8 M urea. Fractions of 7 ml were collected and absorbance at 280 nm was determined for each fraction.
(C) PE-glutenin (20 mg) was dissolved in 0.1 M acetic acid (1 ml) and applied on a Sephadex G-150 column (1.25 x 100 cm) equilibrated with 0.1 M acetic acid. Elution was performed with 0.1 M acetic acid. Fractions of 1 ml were collected and absorbance at 280 nm for each fraction was determined.

V-1, it appears that glutenin contained aggregative subunit polypeptides, which associate in 0.1 M acetic acid, and such aggregation is due to hydrogen, hydrophobic and ionic bonds, primarily hydrogen bond.

Subunit Polypeptide Composition of Each Fraction Obtained by Gel Filtration of CE- or PE-glutenin.

In the previous section, subunit polypeptides of CE- or PE-glutenin were separated by the gel filtration in the presence or absence of urea (Fig. V-1). Subunit polypeptide compositions of each resulting fraction were analyzed by SDS-PAGE. Fig. V-2 shows the elution pattern of CE-glutenin obtained by gel filtraion using acetic acid as an eluant (which is identical with the pattern of Fig. V-1-A) and the SDS-PAGE pattern of each fraction. F-II and F-III fractions contained primarily high mol wt subunits (B-1, 2 and 3) and low mol wt subunits (B-5 , 6 and 7), respectively.[6,63] On the other hand, F-I was composed of B-4, B-5, B-6, B-7 and the broad band in high mol wt region named as "aggregative band". Hence, the polypeptides of F-I fraction were revealed to be aggregative polypeptides. Of these polypeptides, B-5, 6 and 7 seem to be involved in the other aggregative polypeptides, since their mobilities

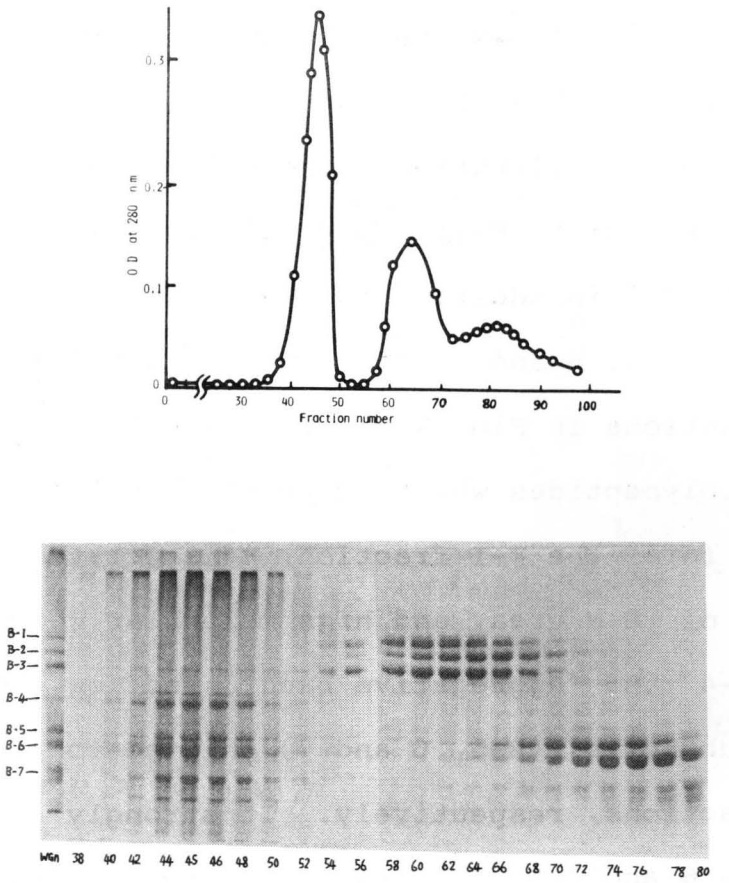


Fig. V-2. Elution Profile of CE-Glutenin on Sephadex G-150 in 0.1 M acetic acid and SDS-PAGE of Each Fraction.

Elution profile of CE-glutenin on sephadex G-150 in 0.1 M acetic acid was the same one as shown in Fig. V-1-A. After lyophilization, each fraction was solubilized in SDS buffer of Laemmli system and applied to SDS-PAGE without 2-ME. "WGn" indicated the pattern of the reduced glutenin.

on SDS-PAGE were identical to those of B-5, 6 and 7 of F-III fraction, respectively.

Fig. V-3 shows the subunit polypetide composition of two peakes (F-II and F-III) of CE-glutenin obtained by gel filtration using 0.1 M acetic acid - 8 M urea as an eluant. F-II newly contained B-4 and "aggregative band" in addition to B-1, 2 and 3. F-III was composed of B-5, 6 and 7 which were detected in F-I and F-III fractions in Fig. V-2. This result suggests that subunit polypeptides which aggregated in 0.1 M acetic acid to form the F-I fraction, dissociated in the presence of 8 M urea, and high molecular weight subunits (B-4 and "aggregative band") and low molecular weight sububnits (B-5, 6 and 7) shifted to F-II and F-III fractions, respectively. It strongly indicates that B-5, 6 and 7 of F-I fraction were involved in the other aggregative polypeptides.

Fig. V-4 shows the subunit compositions of three fractions of PE-glutenin (eluant was 0.1 M acetic acid). F-II and F-III were composed of high mol wt subunits (B-1, 2 and 3) and low mol wt subunits (B-5, 6 and 7), respectively, in the same manner as F-II and F-III of CE-glutenin as shown in Fig. V-2. On the other hand, F-I contained less amount of B-5, 6 and 7, and was

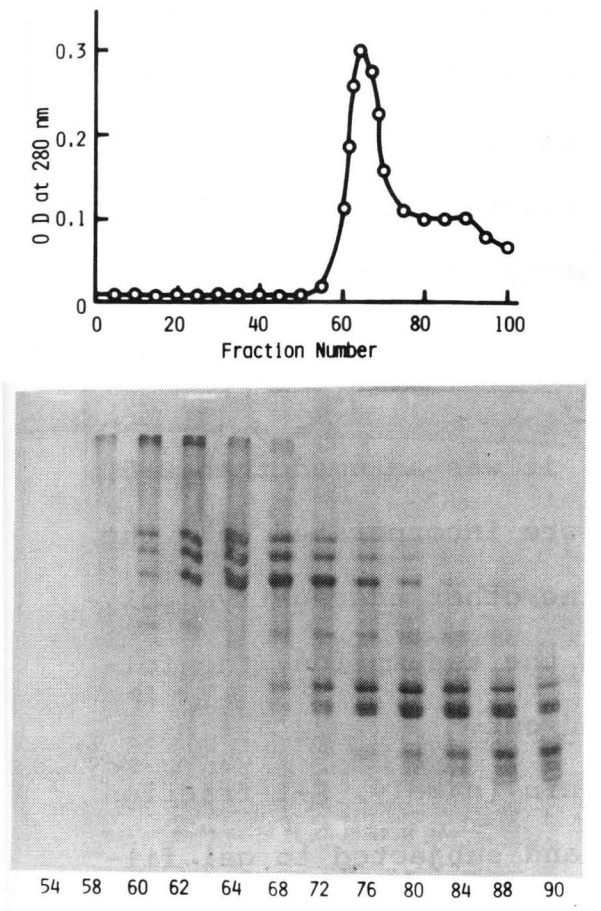


Fig. V-3. Elution Profile of CE-Glutenin on Sephadex G-150 in Acetic Acid - Urea and SDS-PAGE of Each Fraction.

Elution profile was the same as shown in Fig. V-1-B. Each fraction was solubilized in buffer of the Laemmli system and applied to SDS-PAGE without 2-ME.

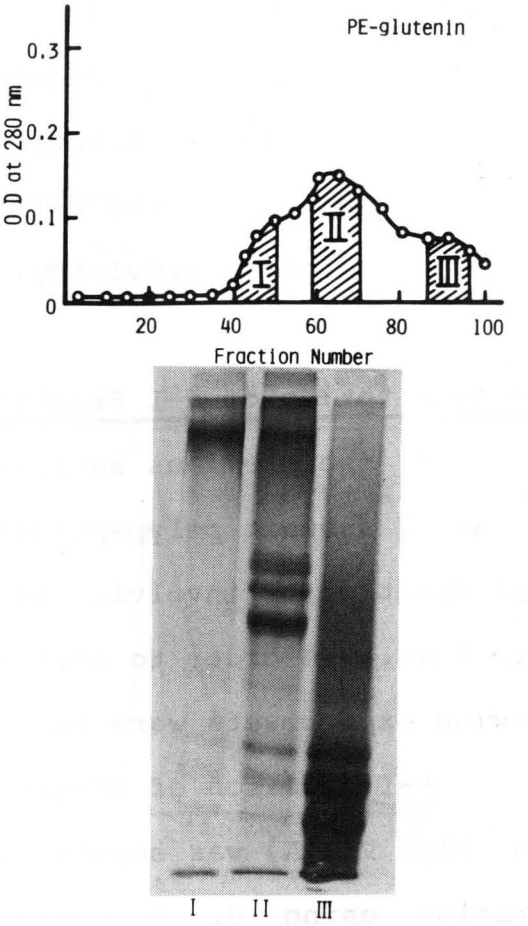


Fig. V-4. Elution Profile of PE-Glutenin on Sephadex G-150 in Acetic Acid and SDS-PAGE of Main Fractions.

Elution profile was the same as shown in Fig. V-1-C. Main fractions, I, II and III were collected and lyophilized. These fractions were solubilized in SDS buffer of the Laemmli system and applied to SDS-PAGE without 2-ME.

composed of primarily B-4 and "agregative band". This indicates that the aggregation of B-5, 6 and 7 subunit polypeptides in F-I fraction was dissociated and these subunits shifted to the F-III fraction with an addition of plus charge (pyridylethyl group) to glutenin.

Reconstitution of F-I Fraction.

In the previous section, it was assumed that B-5, 6 and 7 subunit polypeptides are incorporated into the F-I fraction by involving in the other aggregative polypeptides. In order to clarify the assumption, the following experiments were carried out.

F-I fraction of CE-glutenin (namely, F-I fraction in Fig. V-2-A) was separated and subjected to gel filtration using 0.1 M acetic acid as an eluant (Fig. V-5-A), indicating that F-I fraction remained as one peak in 0.1 M acetic acid. Howevr, gel filtration using 8M urea as an eluant resulted in separating F-I fraction into subfraction II (Sub-II) and subfraction III (Sub-III) as shown in Fig. V-5-B. SDS-PAGE analysis revealed that Sub-II primarily contained "aggregative band" and B-4, and Sub-III primarily consisted of B-1, 2 and 3 subunit polypeptides. The obtained Sub-II and SUb-III were dialyzed against 0.1 M acetic acid to

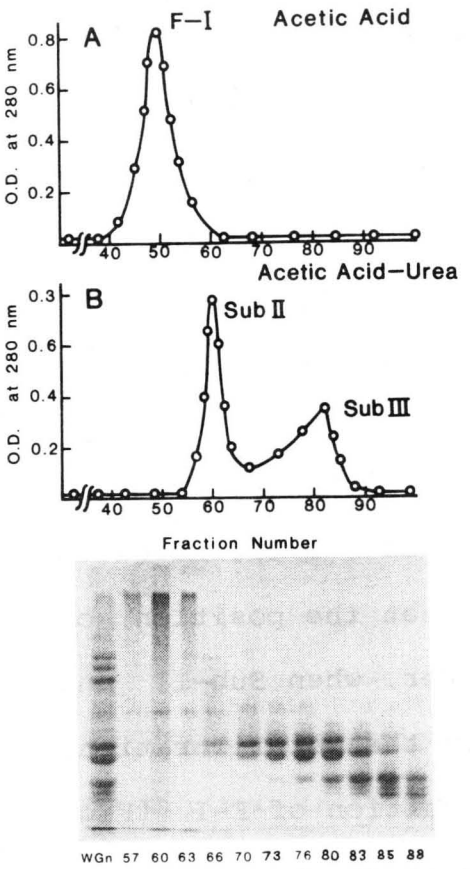


Fig. V-5. Gel Filtration of F-I Fraction on Sephadex G-150.

(A) F-I fraction of CE-glutenin, as shown in Fig. V-1-A, was collected and lyophilized. The lyophilized F-I fraction was dissolved in 0.1 M acetic acid and was applied on a Sephadex G-150 column. Elution was performed with 0.1 M acetic acid. (B) The lyophilized F-I fraction was dissolved in 0.1 M acetic acid - 8 M urea and applied on a Sephadex G-150 column. Elution was performed with 0.1 M acetic acid - 8 M urea. Each fraction was collected and applied to SDS-PAGE as described in Fig. V-3.

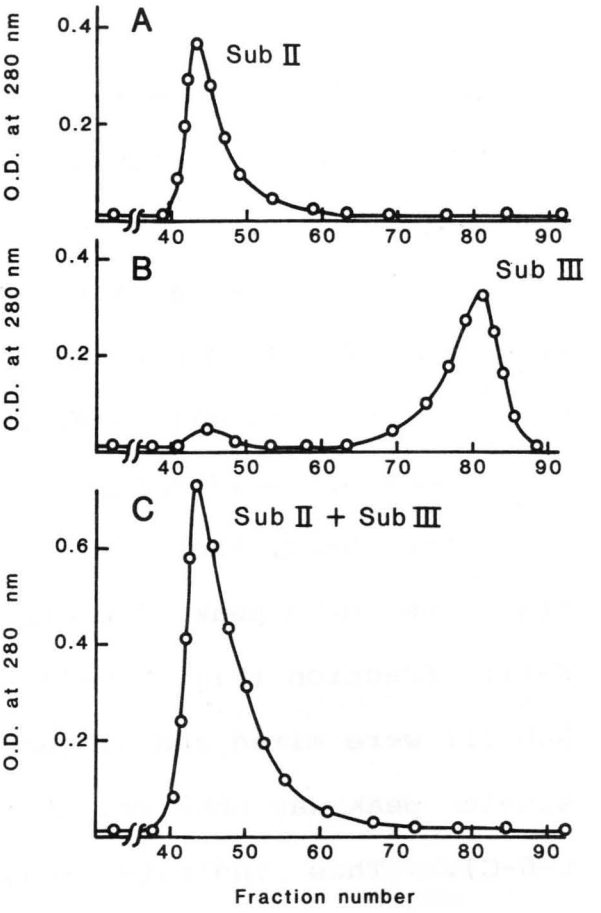


Fig. V-6. Reconstitution of F-I Fraction from Sub II and Sub III.

(A) Sub II of F-I, as shown in Fig. V-5-B, was collected and dialyzed against 0.1 M acetic acid. After lyophilization, Sub II was dissolved in 0.1 M acetic acid and applied on a Sephadex G-150 column. Elution was performed with 0.1 M acetic acid. (B) Sub III of F-I, as shown in Fig. 5-B, was applied on a Sephadex G-150 column as described in (A). (C) Lyophilized Sub II and Sub III were mixed and dissolved in 0.1 M acetic acid. This solution of Sub-II and Sub-III was applied on a Sephadex G-150 column. Elution was performed with 0.1 M acetic acid.

remove urea, and lyophilized to be used in the following experiments. Sub-II and Sub-III subfractions were dissolved in 0.1 M acetic acid separately or together, and were subjected to the gel filtration using 0.1 M acetic acid as an eluant as shown in Fig. V-6. When only Sub-II was subjected to the gel filtration, the peak of protein was observed only at the position of F-I fraction (void volume) as shown in Fig. V-6-A. On the other hand, the gel filtration of Sub-III revealed that the main peak of protein was at the position of F-III fraction (Fig. V-6-B). However, when Sub-II and Sub-III were mixed and subjected to the gel filtration, single peak was observed at the position of F-I (Fig. V-6-C). This indicates that F-I fraction was reconstituted from Sub-II and Sub-III. Results shown in Fig. V-5 and Fig. V-6 totally elucidated as follows; Of subunit polypeptides contained in F-I fraction, B-5, 6 and 7 little aggregate for themselves. However, B-4 and "aggregative band" can form the "core portion" for making the aggregation with B-5, 6 and 7, resulting in the production of F-I fraction.

Proposed Model of Glutenin Molecule Including Subunit-Subunit Interaction through Non-Covalent Forces.

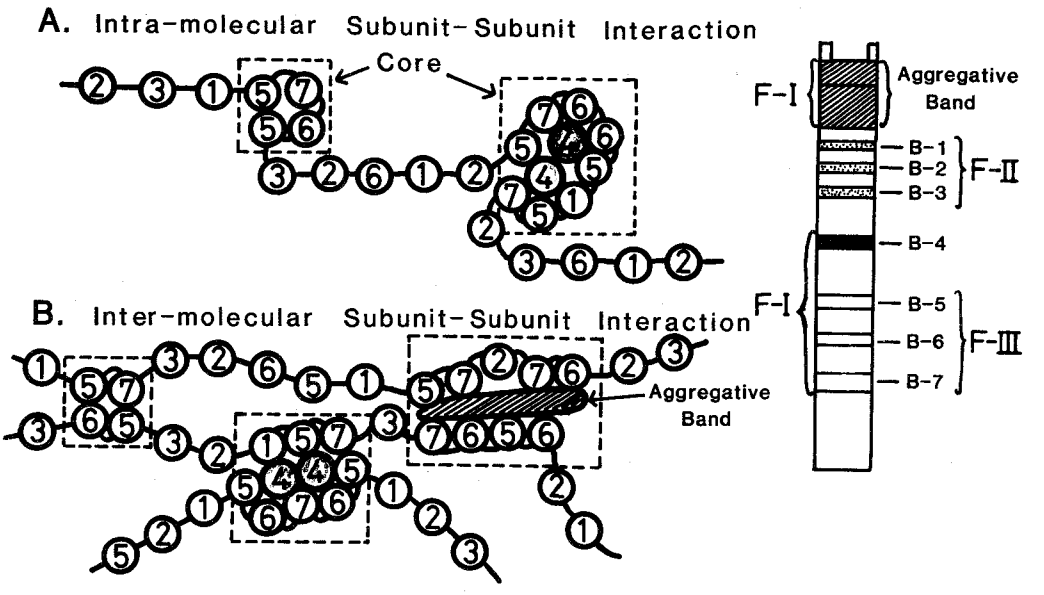


Fig. V-7. Whole Structural Model of Glutenin Molecule Including Subunit-Subunit Interaction through Non-Covalent Forces.

Numbers in circle indicate the kinds of subunit polypeptides of glutenin. (A) Structural model of glutenin molecule including intra-molecular subunit-subunit interaction through non-covalent forces. (B) Structural model of glutenin molecules including inter-molecular subunit-subunit interaction through non-covalent forces.

From the evidence described above, the author proposes the structural model of glutenin molecule including subunitsubunit interaction through non-covalent forces as illustrated in Fig. V-7. Glutenin is primarily a polymer of several kinds of subunit polypeptides linked "linearly" through two inter-polypeptide SS bonds. Howęver, since hydrophobic amino acids and glutamine contents are very high in glutenin,

the interaction between the subunit polypeptides can also occur through non-covalent forces such as hydrophobic and hydrogen bonds. The results in the present chapter elucidated that of the subunit polypeptides of glutenin, especially, B-4 and "aggregative band" associate through non-covalent forces to form the aggregates easily. B-4 and "aggregative band" can also incorporate other non-aggregative subunits (B-5, 6 and 7) into their aggregates. Thus, aggregative subunits (B-4 and "aggregative band") may form the "core portion" for making the aggregation with the other subunit polypeptides in glutenin molecule as shown in Fig. V-7-A. Such "core portions" may be formed between glutenin molecules as well as in one molecule, which probably causes the formation of complicated three-dimensional structure of glutenin molecules as shown in Fig. V-7-B. Such net work structure of glutenin molecules maintained through both SS bonds and non-covalent forces, may primarily contribute the unique functional properties of wheat flour "dough".

CHAPTER VI

Summary

Chapter II

The main subunits of glutenin were separated by preparative SDS-PAGE system (U.K. Laemmli, Nature, 227, 680 (1970)) and their Cys contents were determined by amino acid analysis. Amino acid compositions of glutenin subunits, determined in the present study, were different from those determined by Danno et al. (G. Danno, K.Kanazawa and M.Natake, Agric. Biol. Chem., 40, 739 (1976)). We found that these differences were due to the different methods of hydrolysis of subunit polypeptides. That is, hydrolysis of subunit polypeptides extracted from gel and hydrolysis of polypeptides in gel without extraction. Cys contents of glutenin subunits were determined as (PE-Cys). Although no PE-Cys was detected in B-4 or B-4', all other subunits were shown to have 4 mole Cys per mole protein.

Chapter III

Glutenin was reduced with various concentrations of (2-ME) at pH 6.0 and the liberation of subunits was observed by SDS-PAGE. The amount of SH groups in pro-

tein liberated by reduction increased steeply with the increase of 2-ME concentration around 1 mM. But the increase of SH groups in protein declined at higher concentrations to give a plateau at 6 mM. The level of this plateau, which may correspond to the amount of reactive SS bonds, was 40 % of the total SS bonds of glutenin. In order to investigate whether inter-polypeptide SS bonds are cleaved by the partial reduction, the libration of subunit monomers was observed by SDS-PAGE. By the partial reduction, B-1 + 1' (MW 104 Kd), B-2 (MW 90 Kd), B-3 (MW 81 Kd) and B-7 (MW 35 Kd) appeared in the electrophoretic pattern even at low concentrations of 2-ME, indicating the liberation of these subunits. However, although all monomers of B-7 subunits were almost liberated at low concentrations of 2-ME (0.5 mM), B-1 + 1', 2 and 3 did not show sufficient amounts of monomers even at 6 mM. Further, another groups of bands, which had molecular weights of 136 Kd, 132 kd and 110 Kd, were observed at low concentrations of 2-ME but dissapeared at extremely high concentrations, indicating these bands must be oligomers of glutenin subunits. These observtions from the SDS-PAGE patterns suggested that the reactivities of inter-polypeptide SS bonds differ according to the

kinds of subunits. B-6 (MW 42 Kd) appeared at the position of MW 38 Kd by reduction with lower concentrations of 2-ME, indicating the retention of intra-polypeptide SS bonds. B-1 + 1', 2, 3 and 7 that always appeared at the same positions independent of the 2-ME concentraions, were separated from partial reduction glutenin (0.5 mM of 2-ME) and their SH and SS determined, in order to know whether these subunits retain intra-polypeptide SS bonds. While the amount of liberated SH was much less than 2 moles per mole protein, the amount of SS bonds was near 2 moles, indicating that the liberated subunits retain intra-polypeptide SS bonds.

Chapter IV

Selective cleavage of inter-polypeptide (inter-subunit) SS bonds of wheat glutenin was carried out by the treatment with 32 mM 2-ME at pH 4.0 and 20^{o}C for 4 hr. The subunits liberated by this partial reduction were shown to have uncleaved intra-polypeptide SS bonds from the following criteria : 1) relative mobility on SDS-PAGE of the subunits with low molecular weight from partial reduced glutenin was larger than that from the completely reduced glutenin, showing the compact con-

formation of subunits by uncleaved intra-polypeptide SS bonds; 2) with further reduction by 2-ME in the presence of 8 M urea, these bands decreased their mobility and shifted to the position of the completely reduced glutenin subunts, indicating the complete unfolding and expansion of the subunit polypeptides which may cause the decrease of the electrophoretic mobility. The amount of cysteine residue involved in inter- and intra-polypeptide SS bonds was determined by selective modification with iodoacetamide and vinylpyridine, respectively. All subunits contained 2 moles inter-polypeptide SS bonds per mole subunit, while the number of intra-polypeptide SS bonds was 2-3 moles per mole subunit. It seems likely that wheat glutenin is a linear polymer protein consisted of several subunits which linked together through SS bonds.

Chpater V

In order to elucidate the mode of interaction between subunit polypeptides of glutenin through non-covalent forces, gel filtration of CE- and PE-glutenin on Sephadex G-150 was performed in acetic acid or urea solution. A part of subunit polypeptides of the CE-glutenin aggregated in the presence of 0.1 M acetic acid

(F-I). The aggregative fraction (F-I) was dissociated to subunit polypeptides in the pesence of 8 M urea. This suggests such association was mainly due to hydrogen and hydrophobic bonds. The introduction of positive charge to glutenin (PE-glutenin) decreased the aggregation of the subunit polypeptides compared with that of CE-glutenin, showing that ionic bond also contributes to the aggregation. SDS-PAGE analysis revealed that the aggregative fraction (F-I) of glutenin subunit polypeptides contained B-4 (MW 60 Kd), B-5 (MW 45 Kd), B-6 (MW 42 Kd), B-7 (MW 35 Kd) and "aggregative band" (broad band in high molecular weight region, above 100 Kd). Of these subunit polypeptides, B-4 and "aggregative band" may form the "core portion" for making the aggregation with the other subunit polypeptides, from reconstitution experiments of F-I and its subfractions in the presence or absence of urea. From the evidence as described above, the author proposed the whole model of glutenin molecule including the "subunit-subunit" interactions through both SS bonds and non-covalent forces.

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